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(71) Applicant: RESEARCH CORPORATION TECHNOLOGIES, INC. [US/US]; 6840 East Broadway Boulevard, Tucson, AZ 85710 (US).

(72) Inventor: SRIVASTAVA, Arun ; 975 West Walnut Street, Indianapolis, IN 46202 (US).

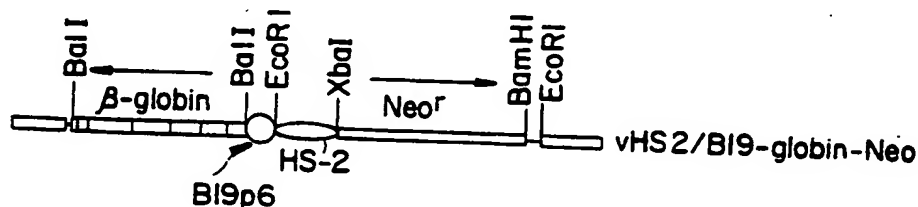
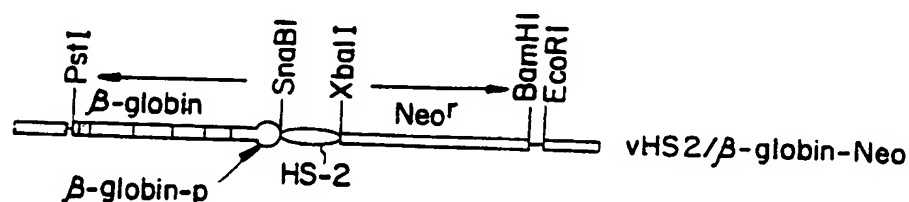
(74) Agents: SCOTT, Anthony, C. et al.; Scully, Scott, Murphy & Presser, 400 Garden City Plaza, Garden City, NY 11530 (US).

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(54) Title: ADENO-ASSOCIATED VIRUS-2 BASAL VECTORS



(57) Abstract

Gene therapy involves the transfer and stable insertion of new genetic information into cells. The present invention is directed to safe vectors for gene therapy and thus provides hybrid parvovirus vectors which are capable of site-specific integration into a mammalian chromosome without substantial cytotoxicity, and which direct erythroid cell-specific expression of heterologous genes. The hybrid vector is useful in gene therapy, particularly in the treatment of hemoglobinopathies and other hematopoietic diseases, and in conferring cell-specific multidrug resistance. A method of delivery of constitutive levels of a pharmaceutical product and a method of producing a recombinant protein are also provided.

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ADENO-ASSOCIATED VIRUS-2 BASAL VECTORS

Gene therapy involves the transfer and stable insertion of new genetic information into cells. The present invention is directed to a safe vector for gene therapy and provides hybrid parvovirus vectors which are capable of site-specific integration into a mammalian chromosome without substantial cytotoxicity, and which can direct cell-specific expression of a desired gene product. The hybrid vectors are useful in gene therapy, particularly in the treatment of hemoglobinopathies. A method of delivery of a pharmaceutical product is also provided. The present invention also provides a method of conferring cell-specific multidrug resistance.

The therapeutic treatment of diseases and disorders by gene therapy involves the transfer and stable insertion of new genetic information into cells. The correction of a genetic defect by re-introduction of the normal allele of a gene encoding the desired function has demonstrated that this concept is clinically feasible [Rosenberg et al. (1990) New Eng. J. Med., 323, 570].

Hematopoietic stem cells or pluripotent progenitor cells are particularly useful for gene therapy studies since, although they are somatic cells, they differentiate to produce all the lineages of blood cells. Hence, the introduction of a foreign gene into a stem or progenitor cell results in the production of various lineages which can potentially express the foreign gene or alter control of native gene products. The introduction of a foreign gene into a progenitor cell or any other appropriate cell requires a method of

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-2-

1 gene transfer to integrate the foreign gene into the
cellular genome. Although a variety of physical and
chemical methods have been developed for introducing
exogenous DNA into eukaryotic cells, viruses have
5 generally been proven to be much more efficient for this
purpose. Several DNA-containing viruses such as
parvoviruses, adenoviruses, herpesviruses and
poxviruses, and RNA-containing viruses, such as
retroviruses, have been used to develop eukaryotic
10 cloning and expression vectors. The fundamental problem
with retroviruses is that they are either the etiologic
agents of, or are intimately associated with,
malignancy. Retroviruses integrate randomly into the
cellular genome, and thus may activate cellular proto-
15 oncogenes or may disrupt sequences critical to cell
function. Accordingly, the use of retroviral vectors in
gene transfer presents a problem in that there is a
finite chance that such vectors may induce neoplasia.
Thus, a need exists for additional and improved vectors
20 for gene transfer.

Whereas retroviruses are frequently the etiologic
agents of malignant disorders, parvoviruses constitute
the sole group of DNA-containing viruses that have not
yet been associated with any malignant disease.
25 Although parvoviruses are frequently pathogenic in
animals, a parvovirus of human origin, the adeno-
associated virus 2 (AAV), has so far not been associated
with any known human disease, even though up to 90% of
the human population has been exposed to AAV.
30 [Blacklow, N. R. (1988) in: Parvoviruses and Human
Disease, CRC Press, Boca Raton]. In addition, most
retroviruses used for gene transfer are of murine

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SUBSTITUTE SHEET

-3-

1 origin, while AAV, a human virus, is physiologically
more relevant for gene transfer in humans. Moreover,
retroviruses are susceptible to inactivation by heat and
organic solvents, whereas AAV is heat stable, extremely
5 resistant to lipid solvents, and stable between pH 3.0
and 9.0. Thus as vehicles for gene transfer,
parvoviruses provide many advantages over retroviruses.

Recombinant retroviruses have low viral titers
(10^5 - 10^6 virions/ml) (Rosenberg) in contrast to the high
10 titers of recombinant AAV (10^8 - 10^9 virions/ml)
[Srivastava et al. (1990) Blood 76, 1997].
Consequently, it is generally not possible to achieve an
infection efficiency with recombinant retrovirus beyond
10-50% of the target cell population, with successful
15 infection requiring actively replicating cells. In
contrast, a 70% infection efficiency has been reported
for a recombinant AAV [Samulski et al. (1989) J. Virol.
63, 3822], and it is possible to achieve a 100%
infectivity of target cells with wild-type AAV [Nahreini
20 et al. (1989) Intervirology 30, 74]. Furthermore, even
though recombinant retroviral vectors have been rendered
replication-incompetent, there remains a low probability
of recombination between the vector and endogenous
retroviral sequences. In contrast, 60-90% of the
25 population is sero-positive for human parvoviruses, and
no endogenous viral sequences have yet to be detected in
volunteer donors. In recombinant AAV vectors, all of
the AAV coding sequences have, nonetheless, been
deleted.

30 Perhaps the most significant advantages of AAV-
based vectors are that they mediate integration into the
host chromosomal DNA in a site-specific and stable

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SUBSTITUTE SHEET

-4-

1 manner. Retroviral genomes, following reverse
transcription, undergo integration into the host
chromosomal DNA with a totally random integration
pattern. AAV establishes a latent infection which is
5 site-specific. The integration site has been mapped to
human chromosome 19. (Kotin et al. (1990) Proc. Natl.
Acad. Sci. USA 87, 2211). It has therefore become
feasible to accomplish site-specific delivery of
exogenous DNA into mammalian cells. While retroviral
10 vectors mediate integration of non-viral sequences into
the host chromosome, the integration pattern is not
always stable. Frequently the integrated retroviral
provirus is excised from the cell. AAV, on the other
hand, establishes a stable integration.

15 Despite the potential advantages outlined above,
the parvovirus-based vectors suffer from one limitation,
and that is the size of a DNA sequence that can be
packaged into the mature virions. For example, whereas
up to 8.0 - 9.0 kilobase pair (kbp) DNA fragments can be
20 packaged into retroviral vectors, a maximum of about 5.0
kbp DNA can be packaged into AAV. This size limitation,
however, does not preclude the cloning and packaging of
most cDNA molecules.

Thus parvovirus-based vectors offer a useful
25 alternative to retroviral vectors for gene therapy in
humans. While AAV-based vectors allow stable, site-
specific integration of transferred genes, the
indiscriminate expression of the transferred gene in all
cell lineages presents significant problems. Thus, a
30 need exists for AAV vectors which effect tissue-specific
expression of the transferred gene. In accordance with
the present invention, one method, for example, to solve

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SUBSTITUTE SHEET

-5-

1 this problem is by a combination of the features of AAV
and another human parvovirus, B19.

While AAV causes no known disease, B19 is known
to be the etiologic agent of a variety of clinical
5 disorders in humans. B19 is the causative agent of
transient aplastic crises associated with various
hemolytic anemias, erythema infectiosum or the "fifth
disease", post-infection polyarthralgia and
thrombocytopenia in adults, and some cases of chronic
10 bone marrow failure and hydrops fetalis.

AAV is dependent on a helper virus, such as
adenovirus, herpesvirus, or vaccinia virus, for optimal
replication. In the absence of a helper virus, AAV
establishes a latent infection in which the viral genome
15 integrates into chromosomal DNA site-specifically. B19,
on the other hand, is an autonomously replicating virus
that is known to replicate only in human hematopoietic
cells in the erythroid lineage. Both AAV and B19
contain linear, single-stranded DNA genomes, but their
20 genomes show no homology at the nucleotide sequence
level. The nucleotide sequences of both genomes are
known. [Lusby et al. (1980) J. Virol. 34, 402,
Srivastava et al. (1983) J. Virol. 45, 555; Shade et al.
(1986) J. Virol. 58, 921]. The AAV genome contains
25 inverted terminal repeats (ITRs) of 145 nucleotides, 125
nucleotides of which form a palindromic hairpin that
plays a critical role during AAV DNA replication. The
sequences of the ITRs are shown in Fig. 1 and as SEQ ID
NO:1. In latently infected cells, the termini of AAV
30 are at the junction of the cellular sequences and thus
the termini also facilitate integration and rescue.

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SUBSTITUTE SHEET

-6-

1 The remarkable features of the two human
parvoviruses can be combined, for example, in an AAV-B19
hybrid vector, to provide vectors in accordance with the
present invention. The vectors of this invention are
5 particularly useful for gene transfer in bone marrow
cells and other hematopoietic cells. These hybrid viral
vectors mediate site-specific integration as well as
tissue-specific expression of heterologous genes in
hematopoietic cells.

10 The present invention is directed to hybrid
parvovirus vectors capable of site-specific integration
into a mammalian chromosome without substantial
cytotoxicity, and which can direct tissue-specific
expression of a heterologous gene, i.e. a non-parvovirus
15 gene. More particularly, the present invention provides
vectors comprising two inverted terminal repeats of
adeno-associated virus 2 and at least one genetic
cassette comprising a promoter capable of effecting
cell-specific expression operably linked to a
20 heterologous gene wherein the cassette resides between
the two inverted terminal repeats. In a preferred
embodiment, the promoter is the p6 promoter of B19
parvovirus and directs erythroid cell-specific
expression of the heterologous gene.

25 In another aspect of this invention, host cells
transduced by the hybrid vectors of the present
invention are provided.

 Another aspect of the present invention provides
a method of treatment for hematopoietic diseases, in
30 particular hemoglobinopathies, by transducing
hematopoietic stem or progenitor cells with a vector of
a present invention and introducing the transduced cells

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SUBSTITUTE SHEET

-7-

1 into a patient, where the heterologous gene is
expressed.

5 A further aspect of this invention provides a
method for delivery of a pharmaceutical product in a
mammal by transducing hematopoietic stem or progenitor
cells with a hybrid vector of the present invention and
introducing the transduced cells into the mammal. The
heterologous gene is expressed, and the mature red blood
cell provides a vehicle for delivery of the heterologous
10 gene product throughout the bloodstream or to the liver
or spleen.

Yet another aspect of the present invention
provides a method of conferring cell-specific multidrug
resistance by transducing cells with a hybrid vector of
15 the present invention in which the heterologous gene is
a multidrug resistance gene, and introducing the
transduced cells into a mammal. In a preferred
embodiment, the hybrid vector contains the B19p6
promoter, and thus the multidrug resistance phenotype is
20 conferred to erythroid cells.

As used herein, transduction refers to a process
by which cells take up foreign DNA and integrate that
foreign DNA into their chromosomes. Transduction can be
accomplished, for example, by transfection, which refers
25 to various techniques described hereinbelow by which
cells take up DNA, or infection, by which viruses are
used to transfer DNA into cells.

Fig. 1 depicts the nucleotide sequence of an ITR
of the AAV 2 genome.

30 Fig. 2 depicts the nucleotide sequence of B19
from nucleotide number 200 to nucleotide number 424 as
numbered by Shade et al. (1986).

-8-

1 Fig. 3 diagrams the construction of a hybrid
vector of the present invention.

 Fig. 4 demonstrates the site-specific integration
of the wild-type AAV genome into normal human diploid
5 chromosomal DNA by Southern blot analysis.

 Fig. 5 demonstrates the site-specific integration
of the recombinant AAV genome into normal human bone
marrow cells by Southern blot analysis.

 Fig. 6 is a diagram of the construction of
10 recombinant plasmids pWP-7A (Panel A) and pWP-19 (Panel
B).

 Fig. 7 is a diagram of the construction of
recombinant plasmid pWN-1.

 Fig. 8 is a diagram of the construction of
15 recombinant plasmids pWP-21 and pWP-22 (Panel A) and
pWP-16 and pWP-17 (Panel B).

 Fig. 9 demonstrates Southern blot analysis of
rescue and replication of the recombinant neo^r gene in
human cells. Panel A: Rescue from plasmid pWP-8A;
20 Panel B: Rescue from plasmid pWP-21; Panel C: Rescue
from plasmid pWP-22; Panel D: Rescue from plasmid pWP-
16; Panel E: Rescue from plasmid pWP-17. Recombinant
plasmids were transfected separately in adenovirus-
infected human KB cells (Lanes 1, 3, 5, 7, 9), or co-
25 transfected with pAAV/Ad helper plasmid (Lanes 2, 4, 6,
8, 10). m and d denote the monomeric and dimeric forms,
respectively, of the recombinant AAV DNA replicative
intermediates.

 Fig. 10 is a diagram of the construction of a
30 hybrid vector in which the neomycin resistance (Neo^r)
gene is under the control of the B19p6 promoter.

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SUBSTITUTE SHEET

-9-

1 Fig. 11 is a graph depicting cell viability after
AAV-mediated transfer of Neo^r to human hematopoietic
stem cells. Panel A illustrates Neo^r gene expression
under the control of the TK promoter. Panel B
5 illustrates Neo^r gene expression under the control of
the B19p6 promoter.

Fig. 12 depicts recombinant AAV vectors of the
present invention containing selectable genes under the
control of B19p6 promoter and a human erythroid-specific
10 enhancer (HS-2).

Fig. 13 depicts the recombinant AAV vectors of
the present invention which express the human β -globin
gene and the Neo^r gene.

Fig. 14 provides a Southern blot of DNA isolated
15 from vHS2/ β -globin-Neo infected K562 cells.

Fig. 15 provides a Northern blot of RNA from K562
cells infected with vHS2/ β -globin-Neo and vHS2/B19-
globin-Neo.

The present invention relates to hybrid
20 parvovirus vectors which comprise a pair of AAV inverted
terminal repeats (ITRs) which flank at least one
cassette containing a promoter which directs cell-
specific expression operably linked to a heterologous
gene. Heterologous in this context refers to any
25 nucleotide sequence or gene which is not native to the
AAV or B19 parvovirus. In accordance with the present
invention, AAV and B19 coding regions have been deleted,
resulting in a safe, noncytotoxic vector.
Representative heterologous genes are described
30 hereinbelow. The AAV ITRs, or modifications thereof,
confer infectivity and site-specific integration, but
not cytotoxicity, and the promoter directs cell-specific

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SUBSTITUTE SHEET

-10-

1 expression and preferably erythroid cell expression
using the p6 promoter of B19 parvovirus. The hybrid
vectors of the present invention thus provide DNA
molecules which are capable of integration into a
5 mammalian chromosome without substantial toxicity.
These hybrid vectors allow safe integration of the DNA
into the cellular genome, since the portions of the DNA
responsible for replication of the parvovirus have been
deleted, and therefore these vectors cannot self
10 replicate.

In accordance with the present invention, the
hybrid vector comprises a first and second terminal
repeat which flank a promoter linked to a heterologous
gene. The terminal repeats can comprise all or part of
15 the ITRs of AAV. The terminal repeats mediate stable
integration of the DNA sequence into a specific site in
a particular chromosome, e.g. human chromosome 19. The
entire DNA sequence, including the ITRs, the promoter,
and the heterologous gene can be integrated into the
20 cellular genome.

The terminal repeats of the hybrid vector of the
present invention can be obtained by restriction
endonuclease digestion of AAV or a plasmid such as
psub201, which contains a modified AAV genome [Samulski
25 et al. (1987) J. Virol. 61, 3096], or by other methods
known to the skilled artisan, including but not limited
to chemical or enzymatic synthesis of the terminal
repeats based upon the published sequence of AAV. The
ordinarily skilled artisan can determine, by well-known
30 methods such as deletion analysis, the minimum sequence
or part of the AAV ITRs which is required to allow
function, i.e. stable and site-specific integration.

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SUBSTITUTE SHEET

-11-

1 The ordinarily skilled artisan can also determine which
minor modifications of the sequence can be tolerated
while maintaining the ability of the terminal repeats to
direct stable, site-specific integration. Site-specific
5 integration can be assessed, for example, by Southern
blot analysis. DNA is isolated from cells transduced by
the vectors of the present invention, digested with a
variety of restriction enzymes, and analyzed on Southern
blots with an AAV-specific probe. A single band of
10 hybridization evidences site-specific integration.
Other methods known to the skilled artisan, such as
polymerase chain reaction (PCR) analysis of chromosomal
DNA can be used to assess stable integration.

The vectors of the present invention contain a
15 promoter which directs tissue-specific expression. For
example, the wild-type parvovirus B19 has a limited host
range and exhibits a remarkable tissue tropism for the
erythroid elements of bone marrow. In a preferred
embodiment, the hybrid vectors of the present invention
20 utilize a transcriptional promoter of B19 to effect
tissue-specific expression of heterologous sequences.
In a more preferred embodiment the promoter is the p6
promoter of B19, which is active in erythroid progenitor
cells. The nucleotide sequence of B19 from nucleotide
25 number 200 to nucleotide number 424 as numbered by Shade
et al. (1986) contains the p6 promoter and is depicted
in Fig. 2 and as SEQ ID NO:2.

The consensus promoter-like sequence TATATATA is
present at nucleotide 320 in B19 (as numbered by Shade
30 et al.) and thus transcription is likely to originate
about 30 nucleotides downstream. It has been discovered
in accordance with the present invention that B19

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SUBSTITUTE SHEET

-12-

1 fragments containing these sequences direct expression
that is specific for erythroid progenitor cells, and
that deletion of B19 coding sequences downstream from
the promoter prevents replication of B19. As explained
5 above, one of ordinary skill in the art can determine
the minimum sequence and modifications of the p6
promoter which provide cell-specific, non-cytotoxic
expression. This can be determined by infecting
erythroid and non-erythroid cells with vectors
10 containing the B19p6 promoter and assessing expression
of the heterologous gene. The promoter sequence can be
derived by restriction endonuclease digestion of B19 or
a cloned B19 plasmid such as pYT103 and pYT107 [Cotmore
et al. (1984) Science 226, 1161] or by any other methods
15 known to the skilled artisan, including but not limited
to chemical or enzymatic synthesis based upon the
published sequence of B19. Other cell-specific
promoters can be obtained by analogous methods, and the
specificity of these promoters is determined by
20 assessing expression in the appropriate cell type.

The promoter of the hybrid vector is operably
linked to the heterologous gene. Any gene that can be
transcribed in such a construction is contemplated by
the present invention. In a preferred embodiment, the
25 heterologous gene encodes a biologically functional
protein, i.e. a polypeptide or protein which affects the
cellular mechanism of a cell in which the biologically
functional protein is expressed. For example, the
biologically functional protein can be a protein which
30 is essential for normal growth of the cell or for
maintaining the health of a mammal. The biologically
functional protein can also be a protein which improves

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-13-

- 1 the health of a mammal by either supplying a missing
protein, by providing increased quantities of a protein
which is underproduced in the mammal or by providing a
protein which inhibits or counteracts an undesired
5 molecule which may be present in the mammal. The
biologically functional protein can also be a protein
which is a useful protein for investigative studies for
developing new gene therapies or for studying cellular
mechanisms.
- 10 The biologically functional protein can be a
protein which is essential for normal growth or repair
of the human body. The biologically functional protein
may also be one which is useful in fighting diseases
such as cancer, atherosclerosis, sickle-cell anemia and
15 the thalassemias. Examples of such biologically
functional proteins are hemoglobin (α , β or γ -globin),
hematopoietic growth factors such as granulocyte-
macrophage colony stimulating factor (GM-CSF),
macrophage colony stimulating factor (M-CSF),
20 granulocyte colony stimulating factor (G-CSF) and
erythropoietin (EPO). Another example is tumor necrosis
factor (TNF), which is a molecule that can be used to
treat cancer, and in particular, tumors. The tumor
suppressors p53 and retinoblastoma (RB) are also
25 contemplated. Various cytokines such as mast cell
growth factor (MGS) and interleukins 1-11 are also
proteins which are contemplated by the present
invention. The biologically functional protein may also
be a selectable marker for antibiotic resistance such as
30 a selectable marker for neomycin resistance in
eukaryotes. Other types of selectable markers such as
adenine phosphoribosyl transferase (APRT) in APRT-

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SUBSTITUTE SHEET

-14-

1 deficient cells, or the firefly luciferase gene are also
included. The heterologous genes encoding these
proteins can be provided by any of a variety of methods,
such as routine cloning procedures (Sambrook et al.),
5 excision from a vector containing the gene of interest,
or chemical or enzymatic synthesis based on published
sequence information. In many instances the DNA
encoding the protein of interest is commercially
available.

10 The biologically functional protein can affect
cellular mechanism by providing a new or altered
function to a cell. For example, the heterologous gene
can be a multidrug resistance gene (mdr) which encodes
P-glycoprotein. P-glycoprotein is a cell membrane
15 glycoprotein which affects intracellular drug
accumulation and is responsible for the phenomenon of
multidrug resistance. (for review, see Biedler [1992]
Cancer 70 1799)

In another embodiment the heterologous gene can
20 encode a non-biologically functional protein. For
example, a hybrid gene comprising various domains and
functions from a variety of sources can be designed and
produced by recombinant technology or enzymatic or
chemical synthesis.

25 In another preferred embodiment the heterologous
gene is capable of being transcribed into an RNA
molecule which is sufficiently complementary to
hybridize to an mRNA or DNA of interest. Such an RNA
molecule is hereinafter referred to as antisense RNA,
30 and has utility in preventing or limiting the expression
of overproduced, defective, or otherwise undesirable
molecules. The vector of the present invention can

35

-15-

1 comprise, as the heterologous gene, a sequence encoding
an antisense RNA which is sufficiently complementary to
a target sequence such that it binds to the target
sequence. For example, the target sequence can be part
5 of the mRNA encoding a polypeptide such that it binds to
and prevents translation of mRNA encoding the
polypeptide. In another embodiment, the target sequence
is a segment of a gene that is essential for
transcription such that the antisense RNA binds the
10 segment (e.g. a promoter or coding region) and prevents
or limits transcription. Hence, the antisense RNA must
be of sufficient length and complementarity to prevent
translation of its target mRNA or transcription of its
target DNA.

15 In a preferred embodiment the antisense RNA is a
15mer and exhibits 100% complementarity to the target
sequence. One of ordinary skill in the art can
determine longer or shorter antisense molecules having
sufficient complementarity to a target sequence such
20 that the antisense molecule is capable of binding to the
target and thereby inhibiting translation or
transcription. The heterologous gene can be provided,
for example, by chemical or enzymatic synthesis, or from
commercial sources.

25 It is preferable that the length of the
heterologous gene is such that the overall size of the
hybrid vector is about 5 kilobases (kb), since the
packaging limit of AAV virions is about 5 kb (Hermonat
et al. (1984) Proc. Natl. Acad. Sci. USA 81, 6466).

30 The hybrid vectors of the present invention can
be provided by inserting the heterologous gene and the
cell-specific promoter between a pair of AAV-derived

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SUBSTITUTE SHEET

-16-

1 terminal repeats. The combination of a promoter and
heterologous gene is also referred to herein as a
cassette. Thus, the invention provides a vector in
which: 1) the terminal repeats mediate stable, site-
5 specific integration into the cellular genome; and 2)
the promoter mediates cell-specific expression of a
heterologous gene, e.g. in erythroid cells, or the
promoter mediates transcription of an antisense RNA or a
sense RNA encoding a polypeptide of interest. The
10 promoter sequence is operably linked to the heterologous
gene in a manner to effect expression of the gene.
Hence, the promoter sequence can be at either or both
ends of the heterologous sequence or coding region.
Furthermore, more than one promoter and heterologous
15 gene can be present in one vector, i.e. there can be two
or more cassettes between the ITRs. Accordingly, more
than one heterologous gene can be expressed by one
vector.

Standard techniques for the construction of such
20 hybrid vectors are well-known to those of ordinary skill
in the art and can be found in references such as
Sambrook et al. (1989) in Molecular Cloning: A
Laboratory Manual, Cold Spring Harbor, New York, or any
of the myriad of laboratory manuals on recombinant DNA
25 technology that are widely available. A variety of
strategies are available for ligating fragments of DNA,
the choice of which depends on the nature of the termini
of the DNA fragments and can be readily determined by
the skilled artisan.

30 It is further contemplated in accordance with the
present invention to include in the hybrid vectors other
nucleotide sequence elements which facilitate

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-17-

1 integration of DNA into chromosomes, expression of the
DNA, and cloning of the vector. For example, the
presence of enhancers upstream of the promoter or
terminators downstream of the coding region can
5 facilitate expression. In another example, recent
studies have identified a DNaseI-hypersensitive site
(HS-2) upstream of the human globin gene cluster that
significantly enhances the erythroid-specific expression
of the globin genes. [Tuan et al. (1985) Proc. Natl.
10 Acad. Sci. USA 82, 6384]. In the hybrid vectors of the
present invention, the presence of HS-2 upstream of the
B19p6 promoter enhances tissue specific expression.

As described hereinabove, the vectors of the
present invention can be constructed by a variety of
15 well-known methods, and the order of the ligation of the
elements can be varied. In a preferred embodiment the
cell-specific promoter and heterologous gene are ligated
together to provide a cassette which can be inserted
between two AAV-ITRs. For example, to provide a
20 cassette containing the B19p6 promoter and a
heterologous gene, a fragment containing the p6-promoter
is inserted into a pUC19 plasmid, after which the p6
containing plasmid is linearized by restriction enzyme
cleavage downstream of the p6 promoter. The
25 heterologous gene is then inserted immediately
downstream of the p6 promoter. A fragment containing
both the p6 promoter and the heterologous gene is
excised from the plasmid and inserted between the AAV-
ITRs in an AAV plasmid from which the AAV coding regions
30 have been deleted. The resulting plasmid comprises the
p6 promoter and a heterologous gene flanked by a pair of
AAV-ITRs. This construction is described more

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SUBSTITUTE SHEET

-18-

1 specifically as follows and is diagrammed in Fig. 3. To
generate a plasmid containing p6, a fragment containing
the p6 promoter of B19 is isolated from B19 DNA or
cloned B19 DNA [see, for example, Cotmore et al. (1984);
5 Shade et al. (1986)]. In a preferred embodiment this
B19 fragment corresponds to nucleotides 200 to 480 as
numbered by Shade et al. (1986) and contains the entire
5' non-coding region and p6 promoter of B19. This 280
bp fragment is flanked by EcoRI and XbaI restriction
10 sites and can be generated by cleavage with these
restriction enzymes. This fragment is cloned into the
EcoRI-XbaI sites of pUC19 to generate plasmid pB19p6.
The skilled artisan will recognize that other plasmids
and restriction sites can be utilized to generate a
15 vector comprising a B19p6 promoter. Alternatively, the
B19p6 promoter can be synthesized chemically or
enzymatically based upon the published sequence and
ligated to the heterologous gene.

A heterologous gene can be operably linked
20 downstream of the B19p6 promoter fragment as follows.
The plasmid pB19p6 is cleaved with HincII, which cleaves
B19 DNA downstream of the p6 promoter (i.e. at
nucleotide 424) and also in the multiple cloning site of
pUC19. The desired heterologous gene is blunt-end
25 ligated downstream from the B19p6 promoter between the
two HincII sites to generate a plasmid pB19p6-insert.
The ordinarily skilled artisan will recognize a variety
of methods, as exemplified, e.g. in Sambrook et al.
(1989), to ligate a fragment containing a cell-specific
30 promoter with a fragment containing the heterologous
gene. In accordance with the present invention, the
coding sequence of GM-CSF, APRT, neo^r, the

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SUBSTITUTE SHEET

-19-

- 1 retinoblastoma gene, α -globin, β -globin and γ -globin
have been employed as the heterologous gene, resulting
in the construction of hybrid vectors, designated AAV-
B19-GM-CSF, AAV-B19-APRT, AAV-B19-neo^r, AAV-B19-RB, AAV-
5 B19- α -globin, AAV-B19- β -globin and AAV-B-19-globin,
respectively. A multidrug resistance gene encoding P-
glycoprotein is also specifically contemplated as the
heterologous gene. The coding sequences of the
respective genes are known [Lee *et al.* (1985) Proc.
10 Natl. Acad. Sci. USA 82, 4360 (GM-CSF); Broderick *et al.*
(1987) Proc. Natl. Acad. Sci. USA 84, 3349 (APRT);
Tratschin *et al.* (1985) Mol. Cell. Biol. 5, 3251 (Neo^r);
Huang *et al.* (1988) Science 242, 1563 (RB-1); Liebhaber
et al. (1980) Proc. Natl. Acad. Sci. USA 77, 7054 (α -
15 globin); Lawn *et al.* (1980) Cell 21, 647 (β -globin);
Enver *et al.* (1989) Proc. Natl. Acad. Sci. USA 86, 7033
(γ -globin) Roninson *et al.* (1986) Proc. Nat'l Acad. Sci.
USA 83, 4538; Roninson *et al.* (1991) in Molecular and
Cellular Biology of Multidrug Resistance in Tumor Cells
20 (ed. Roninson, Plenum Press, NY) 91-106; Schinkel *et al.*
(1991) Cancer Res. 51, 2628; Chen *et al.* (1990) J. Biol.
Chem. 265, 506; (mdr)] and thus can be easily provided
as described hereinabove.

The pB19p6-insert plasmid exemplifies a promoter-
25 heterologous gene cassette which can be isolated by
digesting the plasmid, for example, with EcoRI and
HindIII or other appropriate restriction enzymes, and
then ligated between two AAV-ITRs. The AAV-ITRs are
provided by, for example, restriction digestion of AAV
30 DNA or AAV cloned DNA, or chemical or enzymatic
synthesis based upon the published sequence of AAV ITRs
[Lusby *et al.* (1980)]. In a preferred embodiment, the

-20-

1 AAV ITRs comprise the 145 nucleotides shown in Fig. 1.
Fragments which contain the 125 nucleotides which form
the palindromic hairpin (nucleotide 1-125 of Fig. 1) or
longer fragments which contain the terminal 191
5 nucleotides of the viral chromosome are also useful.
Additional endogenous sequences, for example linkers to
facilitate cloning and ligation, can also be used in the
constructs. In a preferred embodiment, the AAV ITRs are
provided by a plasmid, e.g. p_{sub}201 [Samulski et al.
10 (1987)] which is an AAV derivative into which XbaI
cleavage sites have been introduced at sequence
positions 190 and 4484, and the right-terminal 191 base
pairs of the viral genome have been substituted for the
normal left-terminal 190 base pair domain. This
15 modification results in the extension of the p_{sub}201
terminal repeats to 191 base pairs. The XbaI cleavage
sites allow substitution of the AAV coding region with
exogenous sequences, i.e. the B19 promoter and
heterologous gene, such that the exogenous sequences are
20 flanked by the AAV-ITRs. Derivatives of p_{sub}201
engineered to contain other restriction sites, as
demonstrated in Examples 2 and 3, are also useful for
providing the AAV-ITRs.

To substitute the B19p6-insert, i.e. the
25 cassette, for the AAV coding region, p_{sub}201 is digested
with XbaI to delete the AAV coding regions. Plasmid
vector DNA containing the AAV-ITRs is isolated and
ligated to the B19p6-insert construction. Ligation may
be facilitated by the addition of adapters to the AAV-
30 ITRs and linkers to the B19-p6-insert.

For example, in another preferred embodiment the
vectors of the present invention are constructed by

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SUBSTITUTE SHEET

-21-

1 modifying the engineered XbaI sites of psub201 to
provide additional restriction sites, deleting the AAV
coding regions by digestion with the appropriate
restriction enzyme and ligating the B19p6-insert
5 cassette to the plasmid DNA containing the AAV-ITRs.
The construction of prototype plasmid vectors containing
the AAV-ITRs but not the AAV coding regions, and which
further contain cloning sites to facilitate the
insertion of promoter-heterologous gene cassettes, are
10 exemplified in Examples 2 and 3.

The resulting plasmid comprises a cell-specific
promoter upstream of a heterologous sequence, both of
which are flanked by AAV-ITRs. The order of the
ligations, the nature of the complementary ends, the use
15 of linkers and adapters, and other details can be varied
as necessary by one of ordinary skill in the art to
provide the AAV-B19 hybrid vector of the present
invention.

To establish integration of the vector DNA into
20 the chromosome of a host cell, host cells are
transfected with the vector or infected with mature
virions containing the hybrid vectors. Methods of DNA
transfection are well-known to one of ordinary skill in
the art and include, for example, naked DNA
25 transfection, microinjection and cell fusion. More
efficient integration is accomplished by infection with
virions containing the hybrid vectors.

Virions can be produced by coinfection with a
helper virus such as adenovirus, herpes virus or
30 vaccinia virus. Following coinfection of host cells
with the subject vector and a helper virus, virions are
isolated and the helper virus is inactivated. The

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SUBSTITUTE SHEET

-22-

1 resulting helper free stocks of virions are used to
infect host cells. In another embodiment, virions are
produced by cotransfecting helper virus-infected cells
with the vector of the present invention and a helper
5 plasmid. For example, the hybrid construct of the
present invention can be packaged into mature AAV
virions by cotransfection of adenovirus-infected cells
with the vector of the present invention and a plasmid
which provides the parvovirus rep gene and adenovirus
10 termini. An example of such a plasmid is pAAV/Ad, which
contains the entire coding sequence of AAV and the
adenovirus type 5 terminal sequences in place of the
normal AAV termini. [Samulski et al. (1989)].
Following cotransfection, mature virions are isolated by
15 standard methods, e.g. cesium chloride centrifugation
and heated at 56°C for one hour to inactivate any
contaminating adenovirus. The resulting mature virions
contain the vector of the present invention and are used
to infect host cells in the absence of helper virus.

20 Function of the hybrid vectors of the present
invention, i.e. the ability to mediate transfer and
expression of the heterologous gene in a specific cell
type, can be evaluated by monitoring the expression of
the heterologous gene in transduced cells. For example,
25 bone marrow cells are isolated and enriched for
hematopoietic stem cells (HSC), e.g. by fluorescence
activated cell sorting as described in Srivastava et al.
(1988) J. Virol. 62, 3059. HSC are capable of self-
renewal as well as initiating long-term hematopoiesis
30 and differentiation into multiple hematopoietic lineages
in vitro. HSC are transfected with the vector of the
present invention or infected with varying

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SUBSTITUTE SHEET

-23-

1 concentrations of virions containing a subject hybrid
vector and then assessed for the expression of the
heterologous gene.

5 The assay for expression depends upon the nature
of the heterologous gene. Expression can be monitored
by a variety of methods including immunological, histo-
chemical or activity assays. For example, Northern
analysis can be used to assess transcription using
appropriate DNA or RNA probes. If antibodies to the
10 polypeptide encoded by the heterologous gene are
available, Western blot analysis, immunohistochemistry
or other immunological techniques can be used to assess
the production of the polypeptide. Appropriate
biochemical assays can also be used if the heterologous
15 gene is an enzyme. For example, if the heterologous
gene encodes antibiotic resistance, a determination of
the resistance of infected cells to the antibiotic can
be used to evaluate expression of the antibiotic
resistance gene.

20 In addition to assessing that the heterologous
gene is expressed in the appropriate cells, the correct
promoter specificity of the hybrid vectors can be
evaluated by monitoring the expression of the
heterologous gene, or lack of expression, in cells in
25 which the promoter is not expected to be active. For
example, when cells from a naso-pharyngeal cell line,
KB, are transduced with a hybrid vector containing the
B19p6 promoter, the heterologous gene is not expressed,
since the B19p6 promoter is erythroid cell-specific.
30 Detection of the heterologous gene product at levels at
or below the level of untransduced cells confirms that
the B19p6 promoter of the hybrid vector does not direct

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SUBSTITUTE SHEET

-24-

1 expression of the heterologous gene in non-hematopoietic cells.

The hybrid vectors of the present invention are useful for gene therapy. In particular, the vectors of
5 the present invention can direct erythroid cell-specific expression of a desired gene, and thus are useful in the treatment of hemoglobinopathies.

It is contemplated in accordance with the present invention to use the hybrid vector in the treatment of a
10 variety of diseases, including thalassemia, sickle-cell anemia, diabetes and cancer. The heterologous gene can be the normal counterpart of one that is abnormally produced or underproduced in the disease state, for example β -globin for the treatment of sickle-cell
15 anemia, and α -globin, δ -globin or γ -globin in the treatment of thalassemia. The heterologous gene can encode antisense RNA as described hereinabove. For example, α -globin is produced in excess over δ -globin in δ -thalassemia. Accordingly, δ -thalassemia can be
20 treated in accordance with the present invention by gene therapy with a vector in which the heterologous gene encodes an antisense RNA. The antisense RNA is selected such that it binds to a target sequence of the α -globin mRNA to prevent translation of α -globin, or to a target
25 sequence of the α -globin DNA such that binding prevents transcription of α -globin DNA. In the treatment of cancer the heterologous gene can be a gene associated with tumor suppression, such as retinoblastoma gene, the anti-oncogene p53, or the gene encoding tumor necrosis
30 factor.

The use of the hybrid vectors of the present invention for the treatment of disease involves

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-25-

1 transduction of HSC or progenitor cells with the hybrid
vector. Transduction is accomplished by transfection
with the vector or preparation of mature virions
containing the hybrid vectors and infection of HSC or
5 progenitor cells with the mature virions. Transduced
cells are introduced into patients, e.g. by intravenous
transfusion (see, for example, Rosenberg, 1990). HSC or
progenitor cells are provided by obtaining bone marrow
cells from patients and optionally enriching the bone
10 marrow cell population for HSC. HSC can be transduced
by standard methods of transfection or infected with
mature virions for about one to two hours at about 37°C.
Stable integration of the viral genome is accomplished
by incubation of HSC at about 37°C for about one week to
15 about one month. The stable, site-specific integration
and erythroid cell-specific expression is assessed as
described above. After the transduced cells have been
introduced into a patient, the presence of the
heterologous gene product can be monitored or assessed
20 by an appropriate assay for the gene product in the
patient, for example in peripheral red blood cells or
bone marrow of the patient when expression is erythroid
cell-specific. As described hereinabove, the specific
assay is dependent upon the nature of the heterologous
25 gene product and can readily be determined by one
skilled in the art.

For example, β -thalassemia represents a
heterologous group of clinical syndromes that are
inherited as mutated alleles of genes that encode the
30 human β -globin chain. These mutations affect all
aspects of β -globin gene expression including
transcription, splicing, polyadenylation, translation

-26-

1 and protein stability. The hallmark of β -thalassemia is
the marked reduction or total absence of synthesis of
normal adult hemoglobin (HbA; $\alpha_2\beta_2$). Despite
5 significant advances in the understanding of basic
underlying molecular mechanisms of β -thalassemia,
treatment is limited to regular red blood cell
transfusions and iron-chelation therapy. Treatment by
bone marrow transplantation has also been attempted
[Thomas et al. (1982) Lancet, ii, 227], but an effective
10 cure has not been found.

Accordingly, the vectors of the present invention
are useful in the treatment of β -thalassemia. An AAV-
B19 vector is constructed in which the heterologous gene
is the normal human β -globin gene, with the resulting
15 AAV-B19- β -globin vector allowing parvovirus-mediated
transfer, site-specific integration and erythroid cell-
specific expression of the normal human β -globin gene in
human hematopoietic cells.

Abnormal β -globin expression β -thalassemia may
20 result in the overabundance of α -globin mRNA relative to
 β -globin mRNA. The present invention cannot only
provide a normal β -globin gene, as described
hereinabove, but can further be utilized to down-
regulate the production of excess α -globin by providing
25 a vector with an antisense RNA as the heterologous gene.
An AAV-B19 hybrid vector is constructed in which the
heterologous sequence encodes an antisense RNA which is
sufficiently complementary to a region of the mRNA
encoding the α -chain, such that it binds to and prevents
30 translation of the α -globin mRNA, or to a region of the
DNA encoding α -globin such that it binds to and prevents
transcription of the α -globin gene. Hence, the present

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SUBSTITUTE SHEET

-27-

1 invention contemplates gene therapy for β -thalassemia
comprising transduction of hematopoietic stem or
progenitor cells with a hybrid vector encoding normal β -
5 globin chains, or simultaneous transduction with a
vector encoding a normal β -globin chain and a vector
encoding an RNA antisense to α -globin mRNA or DNA.
Alternately, a construction with more than one B19p6
promoter, as described hereinabove, permits coincident
expression of β -globin and antisense α -globin.
10 Accordingly, transduction with a single vector effects
both the provision of a normal β -globin gene and the
down-regulation of excess α -chains. More specifically,
bone marrow cells are transfected with the subject
vectors, and transduced cells are introduced, e.g. by
15 intravenous transfusion, into a patient. The stable
integration of the vector can be assessed by PCR or
Southern blot analysis and the expression of the
heterologous gene can be evaluated by assaying for the
heterologous gene product in the patient's peripheral
20 blood cells or bone marrow cells. As described
previously, the particular assay depends upon the nature
of the heterologous gene product.

The vectors of the present invention are also
useful in conferring cell specific multidrug resistance.
25 An AAV vector is constructed to contain a cell-specific
promoter and a multidrug resistance gene, with the
resulting vector allowing parvovirus-mediated transfer,
site-specific integration and cell-specific expression
of the mdr gene in a selected cell type. In a preferred
30 embodiment, the vector is AAV-B19-mdr, and confers the
multidrug resistance phenotype to erythroid cells.

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-28-

1 Any of the numerous mdr genes known to confer the
mdr phenotype is useful as the heterologous gene. The
mdr genes are known to the ordinarily skilled artisan
and are described, for example, by Roninson et al.
5 (1991) in Molecular and Cellular Biology of Multidrug
Resistance in Tumor Cells (ed. Roninson, Plenum Press,
NY) 91-106. Accordingly, the present invention provides
a method of conferring cell-specific multidrug
resistance which comprises transducing cells with the
10 hybrid vector of the present invention which contains a
cell-specific promoter and an mdr gene. In a preferred
embodiment, the present invention provides a method of
conferring multidrug resistance to erythroid cells of a
patient which comprises obtaining bone marrow or stem
15 cells from a patient, transducing the bone marrow or
stem cells with the AAV-B19 hybrid vector of the present
invention which contains an mdr gene as the heterologous
gene, and reintroducing the transduced cells into a
patient. The expression of the mdr gene can be
20 evaluated by assaying for the mdr gene product, i.e.,
P-glycoprotein, in the patient's peripheral blood cells
or bone marrow cells. For example, P-glycoprotein can
be detected by known immunologic assays with an antibody
against P-glycoprotein. Such antibodies are known and
25 available to the ordinarily skilled artisan, and are
described, for example, by Meyers et al. (1989) Cancer
Res. 49, 3209 and Georges et al. (1990) Proc. Nat'l
Acad. Sci. USA 87, 152. The present method is
particularly useful as an adjunct to cancer chemotherapy
30 in that it effectively results in the protection of
erythroid cells from the effects of chemotherapeutic
agents targeted to other tissues.

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SUBSTITUTE SHEET

-29-

1 Yet another aspect of the present invention
provides a method for delivery of a pharmaceutical
product, a protein or an antisense RNA in a mammal.
Since the normal differentiation of these stem cells
5 results in production of mature erythrocytes, the
transduction of stem cells with the subject vector
ultimately yields a population of circulating, enucleate
vesicles containing the gene product. This method
comprises transducing hematopoietic stem or progenitor
10 cells with the hybrid vector of the present invention
and introducing, e.g. by intravenous transfusion or
injection, the transduced cells into a mammal.
Transduction can be accomplished by transfecting cells
with the hybrid vector by standard methods of infecting
15 cells with mature AAV virions containing the hybrid
vector at about 37°C for about one to two hours. Stable
integration of the recombinant viral genome is
accomplished by incubating cells at about 37°C for about
one week to about one month. Transduced cells are
20 recognized by assaying for expression of the
heterologous gene, as described hereinabove. In this
embodiment, the pharmaceutical product is encoded by the
heterologous gene of the hybrid vector, and can be any
pharmaceutical product capable of being expressed by the
25 hybrid vector. Such products include α , β and γ -globin,
insulin, GM-CSF, M-CSF, G-CSF, EPO, TNF, MGF,
interleukins, the gene product of the retinoblastoma
gene, p53 or adenosine deaminase. Therefore, the
present invention can provide production of constitutive
30 levels of heterologous gene products inside membrane
vesicles, specifically red blood cells, for in situ
treatment of disease. Optionally, the hybrid vector can

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-30-

1 further comprise a sequence which encodes a signal
peptide or other moiety which facilitates the secretion
of the gene product from the erythroid cell. Such
sequences are well-known to one of ordinary skill in the
5 art [see, for example, Michaelis et al. (1982) Ann. Rev.
Microbiol. 36, 435] and can be inserted into the subject
vectors between the promoter and coding region by
methods described herein. This method can be used to
treat a variety of diseases and disorders and is not
10 limited to the treatment of hemoglobinopathies, since
the heterologous gene is constitutively expressed and
can be released from the red blood cell by virtue of a
secretory sequence, or released when red blood cells are
lysed in the liver and spleen.
15 The following examples further illustrate the
present invention.

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SUBSTITUTE SHEET

-31-

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EXAMPLE 1Site-Specific Integration
of the Recombinant AAV Genome

5 Site-specific integration of the AAV genome was confirmed by an approach in which normal human diploid fibroblasts (HDF) were either mock-infected, or infected with an increasing multiplicity-of-infection (moi) of wild-type AAV. Following multiple serial passage of
10 these cells in culture, their total genomic DNA was isolated, digested with a variety of restriction endonucleases, and analyzed on Southern blots using an AAV-specific DNA probe. A representative Southern blot is presented in Fig. 4. Restriction enzymes are
15 indicated at the top of the figure. The moi is indicated at the top of each lane, with 0.0 indicating mock-infection. The predominant single band of hybridization is evidence that the wild-type AAV genome integrates into normal human diploid cell chromosomal
20 DNA in a site-specific manner. The target site was saturated only at very high moi of AAV, and no selection procedure was employed to select for cell populations that have the integrated provirus.

The site-specific integration of the recombinant
25 AAV genome is demonstrated utilizing human bone marrow cells, which are the target cells for therapy of hemoglobinopathies.

Bone marrow cells were obtained from hematologically normal volunteer donors, and low-
30 density, mononuclear bone marrow (LDBM) cells were isolated by Ficoll-Hypaque density centrifugation. LDBM cells were infected with the recombinant AAV-Neo virions

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SUBSTITUTE SHEET

-32-

1 (vSV40-Neo), in which the Neo gene, under the control of
the SV40 early promoter, is encapsidated into AAV
particles, and incubated in the presence of various
cytokines such as GM-CSF (1 ng/ml) and IL-3 (1 ng/ml)
5 for 48 hours. The cells were incubated in liquid
cultures in the presence of G418 at 37°C for 10 days,
their total genomic DNA was isolated, cleaved with
BamHI, and analyzed on a Southern blot using a Neo-
specific DNA probe as shown in Fig. 5. Concentration of
10 G418 is indicated at the top of each lane. The single
band of hybridization indicated by the arrow
demonstrates that the recombinant AAV viral genome
undergoes site-specific integration into human bone
marrow cell chromosomal DNA.

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SUBSTITUTE SHEET

-33-

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EXAMPLE 2Construction of Recombinant
Plasmids pWP-7A and pWP-19

5 The general overall strategy used to construct
the prototype plasmid vectors, designated pWP-7A and
pWP-19, respectively, is depicted in Figure 6. The XbaI
sites in plasmid psub201 were converted to EcoRI sites
by ligating synthetic XbaI-EcoRI-XbaI adaptors as
10 described by Srivastava *et al.* (1989) Proc. Natl. Acad.
Sci. USA 86, 8078. The AAV coding region was removed
following digestion with EcoRI, and the vector DNA
containing the two AAV-ITRs was isolated from
preparative agarose gels (Seth [1984] Gene Anal. Tech.
15 1, 99), and treated with PolIk to generate blunt-ends.
Similarly, pBR322 DNA was cleaved with EcoRI and PvuII
and a 2066 bp fragment containing the entire coding
region of a gene for resistance to tetracycline (Tc^R)
was also blunt ended with PolIk. These two fragments
20 were ligated and used to transform competent E. coli
HB101 cells by the standard methods described in
Sambrook *et al.* (1989) to generate a plasmid, designated
pWP-7A. Since blunt-end ligation of DNA fragments
containing repaired EcoRI and PvuII ends regenerates an
25 EcoRI site, plasmid pWP-7A can be cleaved with EcoRI
downstream from the Tc^R gene for cloning a gene or
cassette of interest. The neo^R gene under the control
of the herpesvirus thymidine kinase (TK) promoter was
isolated from plasmid pSHL-172 (Tratschin *et al.* [1985]
30 Mol. Cell. Biol. 5, 3251) by partial digestion with
PvuII, and blunt-end ligated with PolIk-treated pWP-7A
DNA. The resulting recombinant plasmid, designated pWP-

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SUBSTITUTE SHEET

-34-

1 8A, is shown in Figure 6-A. Plasmid pWP-19 was
constructed as follows. Plasmid pWP-8A was linearized
with HindIII, which cleaves at the 5' end of the Tc^R
gene, and partially digested with XmnI to remove the Tc^R
5 gene. A plasmid pGBOR which contains a gene for
resistance to ampicillin (Ap^R) and the bacteriophage
lambda operator (OR1/OR2; λ o) sequences (Samulski *et al.*
[1991] *EMBO J.* 10, 3941) was cleaved with EcoRI and XbaI
10 and the fragment containing the λ o sequence was blunt-
end ligated with PstI-treated pWP-8A DNA described
above. The resulting recombinant plasmid pWP-19 is
shown in Figure 6-B.

The plasmid vectors pWP-7A and pWP-19 are useful
for constructing recombinant AAV genomes because direct
15 insertion of an insert of interest is possible in both,
and the presence of the built-in neo^R gene in pWP-19
provides a strong selectable marker in human cells. The
plasmid vector pWN-1 is particularly useful because it
offers several features. In bacterial cells, these
20 include: 1) The availability of a variety of cloning
sites (EcoRI, BamHI, SacI, KpnI, XbaI, SalI, AccI,
HincII, PstI, SphI and HindIII), including the NdeI
site; 2) AAV-ITRs which are well separated from the
cloning sites; and 3) the use of Tc^R as well as Ap^R as
25 selectable markers. In mammalian cells, following
transfection in the presence of the AAV helper plasmid
and Ad, the insert of interest, which is now flanked by
the two AAV-ITRs in their proper orientation (see Figure
7), can be efficiently rescued from the Tc^R gene
30 followed by DNA replication and packaging in the AAV
progeny virions.

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SUBSTITUTE SHEET

-35-

1 Whereas plasmid pWP-7A is useful for cloning
 inserts up to 2.5 kb in size at the unique EcoRI site,
 plasmid pWP-19 offers a built-in neo^R marker gene as
 well as a number of cloning sites such as BamHI, SacI
5 and KpnI, and insert up to 2.6 kb in size can be
 inserted between the two AAV-ITRs.

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-36-

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EXAMPLE 3Construction of Recombinant Plasmid pWN-1

5 The strategy to construct the recombinant plasmid
pWN-1 is shown in Figure 7. Briefly, psub201 plasmid
DNA was digested to completion with XbaI and PvuII, and
a 191-bp XbaI-PvuII fragment containing the entire AAV-
ITR sequence was isolated as described in Example 2.
Similarly, pBR322 plasmid DNA was cleaved with EcoRI and
10 AvaI to isolate a 1425-bp fragment that contains the Tc^R
gene but lacks the origin of DNA replication ("ori")
sequence. This fragment was treated with PolIk to
generate blunt ends. Blunt-ended EcoRI-AvaI fragment
was mixed with a large excess of the XbaI-PvuII fragment
15 containing the AAV-ITR, blunt-end ligated using T4 DNA
ligase, and then digested exhaustively with XbaI. This
resulted in the production of the Tc^R gene flanked by a
single AAV-ITR at each end but in the opposite
orientation (see small arrows in boxed ITRs). This
20 fragment was subsequently ligated at the unique XbaI
site in plasmid pGBOR described above, and Tc^R was used
to select for the recombinant plasmid pWN-1.

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SUBSTITUTE SHEET

-37-

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EXAMPLE 4Rescue and Replication of a Cloned
Insert from Recombinant AAV-Based Plasmids

5 DNA sequences flanked by the two AAV-ITRs can be
rescued from the recombinant plasmids of Examples 2 and
3 following transfection in human cells in the presence
of the AAV and Ad proteins as a prelude to successful
packaging of these genes into mature AAV virions. The
10 insert size between the two AAV-ITRs in plasmid pWP-8A
is similar to that of the wt AAV genome. The AAV-rep
gene the parent plasmid psub201, as well as the λ
OR1/OR2 sequences from plasmid pGBOR were isolated and
inserted in plasmid pWP-19 by the strategy shown in
15 Figure 8-A. Two recombinant plasmids, pWP-21 and pWP-
22, were generated which contain the AAV-rep gene in
different orientations with respect to the neo^R gene.
These plasmids are depicted in Figure 8-A. Similarly,
the insert size between the two AAV-ITRs in plasmid pWN-
20 1 was increased by inserting the neo^R gene either at the
NdeI site or at the PstI site to generate two
recombinant plasmids, pWP-16 and pWP-17, respectively,
which are shown in Figure 8-B.

Plasmids pWP-8A, pWP-21, pWP-22, pWP-16 and pWP-
25 17 were either transfected alone, or co-transfected with
the AAV helper plasmid (pAAV/Ad) separately, in Ad-
infected human KB cells (Samulski et al. [1989] J.
Virol. 63, 3822; Srivastava et al. [1989]; Srivastava
[1990] Blood 76, 1997). Low M_r DNA samples isolated by
30 the method described by Hirt (1967) J. Mol. Biol. 26,
365, were digested with DpnI and analyzed on Southern
blots (Southern [1975] J. Mol. Biol. 98, 503) using a

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SUBSTITUTE SHEET

-38-

1 neo-specific DNA probe as previously described (Samulski
et al. 1989). The results are presented in Figure 9.
No rescue/replication of the recombinant neo^r gene from
5 plasmid pWP-8A occurred in the absence of the pAAV/Ad
helper plasmid (Lane 1); successful rescue and
replication indeed occurred when the AAV-Rep proteins
were supplied in trans (Lane 2), as detected by the
presence of the characteristic monomeric and dimeric
10 replicative intermediates of the recombinant AAV genome.
Similarly, rescue and replication occurred from plasmids
pWP-21 (Lanes 3 and 4), and pWP-22 (Lanes 5 and 6) even
in the absence of the helper plasmid because these
plasmids contain the AAV-rep gene in cis. Rescue and
15 replication from plasmids pWP-16 and pWP-17 also
occurred, but only in the presence of the AAV helper
plasmid (Lanes 8 and 10).

Following rescue and replication, the neo^r gene
could also be packaged into mature AAV progeny virions
in presence of the AAV-Cap proteins. The recombinant
20 AAV progeny virions were biologically active and
infectious. For example, recombinant AAV-neo virions
were used to transduce and stably integrate the neo^r
gene in a variety of diploid and polyploid human cells.
The transduced neo^r gene was biologically active, as
25 determined by gene expression analyses on Northern
blots, as well as by ready isolation of clonal
populations of human cells that were resistant to
geneticin.

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SUBSTITUTE SHEET

-39-

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EXAMPLE 5Construction of an AAV-B19 Hybrid
Parvovirus Cloning and Expression Vector

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A hybrid vector containing the AAV-ITRs the B19p6 promoter and the neo^r gene as the heterologous gene was constructed as follows. The general strategy for the construction of this vector, designated pB19-p6-Neo^r-AAV-ITR, is shown in Fig. 10.

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A near full-length B19 DNA clone (for example pYT104v in which B19 is downstream of the bacteriophage SP6 promoter) was cleaved with SacI and DraI, and the small DNA fragment that contains the left ITR was discarded. Following blunt-end re-ligation of the larger fragment, the plasmid DNA was cleaved with EcoRI and XbaI to isolate the 280 bp fragment that contains the entire 5' non-coding region and the p6 promoter of B19. This fragment was cloned into the EcoRI-XbaI sites of pUC19 to generate a plasmid pB19p6. This plasmid was cleaved with HincII which digests the B19 DNA downstream from the p6 promoter and also the pUC19 DNA in the multiple cloning site. The bacterial Neo^r gene (Tratschin *et al.*, 1985) was blunt-end ligated downstream from the B19p6 promoter between the two HincII sites to generate the plasmid pB19p6-Neo^r. This plasmid was digested with EcoRI and HindIII and the B19p6-Neo insert was isolated and ligated between the two AAV-ITRs of XbaI digested psub201. The vector was packaged into mature AAV virions (vB19-Neo) by cotransfection of adenovirus infected cells with

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-40-

1 adenovirus type 5 terminal sequences (Samulski et al.
1989).

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SUBSTITUTE SHEET

-41-

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EXAMPLE 6**Recombinant Parvovirus-Mediated Transfer of
Bacterial Neo^r Gene in Human Hematopoietic Stem Cells**

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Human hematopoietic stem cells were isolated from normal volunteer donors followed by sorting with monoclonal antibodies against the human CD34 and DR antigens according to the method of Lu *et al.* (1987) J. Immunol. **139**, 1823 to produce a CD34⁺DR⁻ cell

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population. This cell population is known to contain several classes of primitive human hematopoietic progenitor cells including colony forming unit-blast cells (CFU-B1), high-proliferative potential colony forming cells (HPP-CFC), and cells responsible for initiating long-term hematopoiesis *in vitro* (LTBMIC).

15

Approximately 1×10^3 CD34⁺DR⁻ cells isolated from two different donors were either mock-infected, or infected at varying moi with vTK-Neo or vB19-Neo virions. (vTK-Neo is recombinant AAV virion containing the Neo^r gene under the control of the thymidine kinase (TK) promoter). Cells were incubated at 37°C for one week in the presence of the cytokines interleukin-3 (1 ng/ml), granulocyte macrophage colony stimulating factor (1 ng/ml), and a factor for c-kit ligand termed mast cell growth factor (50 ng/ml). G418 was added at a final concentration of 250 µg/ml. The total number of viable cells was counted following one-week exposure to the drug. The concentration of G418 was then increased to 500 µg/ml, and viable cell counts were obtained after two weeks for vTK-Neo-infected cells, and after one week for vB19-Neo-infected cells. These data are shown in Fig. 11.

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SUBSTITUTE SHEET

-42-

1 Exposure to vTK-Neo virions resulted in a nearly
10-fold increase in the G418-resistant hematopoietic
cell population compared with mock-infected cells,
whereas the exposure to the vB19-Neo virions resulted in
5 approximately 4-fold increase at the highest moi of the
virions compared with mock infected cells. These
results demonstrate that the B19p6 promoter is active in
cell populations enriched for HSC, albeit at a lower
level compared with the TK promoter.

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SUBSTITUTE SHEET

-43-

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EXAMPLE 7Evaluation of Tissue-Specificity of the B19p6 Promoter

Non-erythroid cells were infected to determine whether the B19p6 promoter in the hybrid constructions had become indiscriminate or had maintained its erythroid specificity.

Human KB cells were either mock-infected or infected separately with equivalent moi of vTK-Neo and 10vB19-Neo. At 48 hours post-infection cells were exposed to various concentration of G418. Following a 14-day incubation period at 37°C, the approximate numbers of G418-resistant colonies were enumerated. A colony is defined as a group of eight or more cells. These data are presented in Table 1, and demonstrate that under conditions of viral infection, the B19p6 promoter retains its erythroid-specificity.

TABLE 1

Approximate Numbers of G418-Resistant Colonies in KB Cells Transduced with AAV-Neo Virions

Recombinant virus	200 µg/ml G418	400 µg/ml G418	600 µg/ml G418
1. None	10-20	0	0
2. vTK-Neo	TMTC*	100-200	50-100
3. vB19-Neo	10-20	0	0

*TMTC = Too many to count

SUBSTITUTE SHEET

-44-

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EXAMPLE 8**Construction of Parvovirus Vectors
with an Erythroid-Specific Enhancer**

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In order to further increase the tissue-specific expression directed by the B19p6 promoter, the DNaseI-Hypersensitive Site-2 (HS-2) of the Locus Control Region (LCR), (Tuan et al. (1985) Proc. Natl. Acad. Sci. USA 82, 6384), an erythroid-specific enhancer, was inserted into the hybrid vectors of the present invention. As diagrammed in Fig. 12, the HS-2 gene is inserted upstream of the B19p6 promoter and luciferase gene to provide the vector vHS2/B19-Luc. Restriction sites used to facilitate vector construction are shown in Fig. 12. These constructs were packaged into mature AAV virions.

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SUBSTITUTE SHEET

-45-

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EXAMPLE 9Construction of Parvovirus Vectors
Containing the Normal Human β -Globin Gene

5 In order to provide vectors for gene transfer in clinical cases of β -thalassemia and sickle-cell anemia, two plasmid vectors that contain the normal human β -globin gene were constructed and packaged into recombinant AAV virions. The pHS2/ β -globin-Neo construct contains the β -globin promoter and the upstream HS-2 enhancer, along with the Neo^r gene under the control of the TK promoter, and the pHS2/B19-globin-Neo construct also contains the B19p6 promoter. These constructs are shown in Fig. 13.

15 The pHS2/ β -globin-Neo vector was constructed as follows. A plasmid (pWP19) was constructed that contains the Neo^r gene under the TK promoter between the two AAV-ITRs. pWP19 was linearized with SacI. A SnaBI-PstI fragment containing the genomic clone of the human β -globin gene was ligated, in reverse orientation, upstream of the TK promoter. The resulting plasmid was linearized by digestion with KpnI, and a HindIII-XbaI fragment containing the HS2 enhancer was ligated upstream of the β -globin promoter.

25 The pHS2/B-19-globin-Neo vector was constructed as follows. First the HindIII-XbaI fragment containing the HS2 enhancer was cloned upstream of the B19p6 promoter in the plasmid pB19p6 by linearizing it with EcoRI. The HS2-B19p6 fragment was isolated by digesting this plasmid with PvuII and HincII. The PvuII-HincII fragment was ligated to pWP19 plasmid linearized with SacI. Second, the β -globin coding region lacking the β -

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-46-

- 1 globin promoter was excised by digesting the plasmid
with NcoI and PstI. This NcoI-PstI fragment was ligated
to the plasmid described above by linearizing it with
KpnI.
- 5 Similarly, vectors containing the human α -globin
gene in both orientations were constructed and packaged
into recombinant AAV virions. A HinfI-PvuII fragment of
the cloned α -globin gene (Liebhaber, 1980) was ligated
downstream from the B19p6 promoter following
- 10 linearization of the pB19p6 plasmid DNA. The resulting
plasmid was linearized with FspI, and ligated with the
pWP19 plasmid at the SacI site prior to packaging into
the AAV virions.

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SUBSTITUTE SHEET

-47-

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EXAMPLE 10AAV Transduction of Human Cells
with the Multidrug Resistance Gene

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To assess the ability of AAV vectors to confer the multidrug resistance phenotype to drug sensitive human cells, drug sensitive human KB cells are transduced with the AAV vector containing an mdr gene under the control of the thymidine kinase promoter. The expression of the mdr phenotype is assessed by detection of P-glycoprotein with an antibody specific for this protein. Further, the ability of the transduced cells to reduce intracellular accumulation of certain anti-tumor drugs is evaluated by culturing transduced cells in a series of tissue culture media containing step-wise concentration increases of the selected drug. An increase in viability of transduced cells relative to control (untransduced) cells is indicative of expression of the mdr gene.

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To evaluate the ability of the AAV vectors of the present invention to confer cell-specific multidrug resistance, both erythroid and non-erythroid (e.g. drug sensitive KB) cells are transduced with the AAV vector containing an mdr gene under the control of the B19p6 promoter (AAV-B19p6-mdr). KB cells are transduced and assessed as described above. Since the B19p6 promoter directs erythroid cell-specific expression, the mdr gene is not expressed by transduction of KB cells with AAV-B19p6-mdr.

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The ability of AAV-B19p6-mdr to confer erythroid cell-specific multidrug resistance is assessed as follows. Bone marrow is removed from a patient,

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SUBSTITUTE SHEET

-48-

- 1 enriched to CD34⁺ cells and transduced with AAV-B19p6-
mdr. Primary cultures are then established and
maintained until focal colonies can be transferred to
tissue culture trays. Outgrowth of such colonies
5 permits the assessment of resistance to a range of anti-
tumor drugs.

The AAV-B19p6-mdr vector is useful in cancer
chemotherapy. For example, bone marrow is removed from
the iliac creast of a cancer patient, and enriched for
10 CD34⁺ cells. Enriched cells are transduced by AAV-
B19p6-mdr and reinfused into the patient.

The B19p6 promoter can be substituted by other
cell-specific promoter elements to permit expression of
the multidrug resistance phenotype in other cell
15 lineages.

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-49-

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EXAMPLE 11

Cells of a human erythroleukemia cell line, K562 (ATCC CCL-243) were infected with vHS2/B19-globin-Neo (see Example 9 and Fig. 13) to assess the ability of the vector to provide expression of the β -globin gene in a host cell which exhibits no β -globin gene expression.

K562 cells were either mock-infected or infected separately with equivalent moi of vHS2/B19-globin-Neo and vHS2/ β -globin-Neo. DNA of infected cells was isolated, digested with NcoI, and subjected to Southern blot analysis. Fig. 14 provides a Southern blot in which Lane 1 corresponds to DNA isolated from mock-infected cells, and Lane 2 corresponds to DNA isolated from cells infected with vHS2/ β -globin-Neo. The blots were probed with a γ -globin probe (left panel) and a β -globin probe (right panel). The arrow denotes the transduced allele of the β -globin gene in K562 cells infected with vHS2/ β -globin-Neo. Similar results were obtained for K562 cells infected with vHS2/B19-globin-Neo.

The β -globin gene is present but not expressed in K562 cells. Northern blots of total RNA from the mock-infected and infected cells described above were probed with a neomycin probe (Fig. 15, left panel) and a β -globin probe (Fig. 15, right panel). The center panel of Fig. 15 presents an ethidium-bromide stained gel. Lane 1 in each panel represents RNA from mock-infected cells; Lanes 2 and 3 correspond to RNA from vHS2/ β -globin-Neo infected cells; Lanes 4 and 5 correspond to RNA from vHS2/B19-globin-Neo infected cells. Plus and minus signs indicate the presence or absence,

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SUBSTITUTE SHEET

1 respectively, of hemin, an inducer of γ -globin which
2 appeared to have no effect on β -globin expression. The
3 Northern analysis indicates that the β -globin gene is
4 expressed in K562 cells infected with vHS2/ β -globin-Neo
5 and vHS2/B19-globin-Neo, but not in mock-infected cells.

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SUBSTITUTE SHEET

-51-

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Srivastava, Arun
- (ii) TITLE OF INVENTION: SAFE VECTOR FOR GENE THERAPY
- (iii) NUMBER OF SEQUENCES: 2
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Scully, Scott, Murphy Presser
 - (B) STREET: 400 Garden City Plaza
 - (C) CITY: Garden City
 - (D) STATE: New York
 - (E) COUNTRY: USA
 - (F) ZIP: 11530
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release q.0, Version q.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: McNulty, William E.
 - (B) REGISTRATION NUMBER: 22,606
 - (C) REFERENCE/DOCKET NUMBER: 8361
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (516) 742-4343
 - (B) TELEFAX: (516) 742-4366
 - (C) TELEX: 230 901 SANS UR

-52-

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 145 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CGACGCCCCG GCTTTGCCCC GGCGGCCTCA GTGAGCGAGC GAGCGCGCAG AGAGGGAGTG	120
GCCAACTCCA TCACTAGGGG TTCCT	145

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 225 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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ATATATAGCA CGGTACTGCC GCAGCTCTTT CTTTCTGGGC TGCTTTTTC TGGACTTTCT	180
TGCTGTTTTT TGTGAGCTAA CTAACAGGTA TTTATACTAC TTGTT	225

1 WHAT IS CLAIMED:

- 5 1. An expression vector for site-specific
integration and cell-specific gene expression comprising
two inverted terminal repeats of adeno-associated virus
2 and at least one cassette comprising a promoter
capable of effecting cell-specific expression wherein
said promoter is operably linked to a heterologous gene,
and wherein said cassette resides between said inverted
10 terminal repeats.
2. The vector of Claim 1 wherein each of said
inverted terminal repeats comprises the nucleotides of
SEQ ID NO:1.
3. The vector of Claim 1 wherein each of said
15 inverted terminal repeats comprises nucleotides 1 to 125
of SEQ ID NO:1.
4. The vector of Claim 1 wherein said promoter
is a B19 parvovirus promoter.
5. The vector of Claim 4 wherein said B19
20 parvovirus promoter is the p6 promoter.
6. The vector of Claim 4 wherein said B19
parvovirus promoter comprises the nucleotides of SEQ ID
NO:2.
7. The vector of Claim 1 wherein said
25 heterologous gene encodes a biologically functional
protein.
8. The vector of Claim 1 wherein said
heterologous gene encodes a non-biologically functional
protein.
9. The vector of Claim 1 wherein said
30 heterologous gene encodes an antisense RNA.

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- 1 10. The vector of Claim 1 wherein said
heterologous gene is selected from the group consisting
of a gene encoding α -globin, β -globin, γ -globin,
granulocyte macrophage-colony stimulating factor (GM-
5 CSF), tumor necrosis factor (TNF), any one of
interleukins 1-11, neomycin resistance, luciferase,
adenine phosphoribosyl transferase (APRT),
retinoblastoma, insulin, mast cell growth factor, p53,
adenosine deaminase.
- 10 11. The vector of Claim 1 wherein said
heterologous gene encodes P-glycoprotein.
12. The vector of Claim 9 wherein said antisense
RNA is complementary to a segment of the DNA or RNA
encoding α -globin.
- 15 13. The vector of Claim 5 wherein said vector is
AAV-B19-GM-CSF, AAV-B19-APRT, AAV-B19-neo^r, AAV-B19-RB,
AAV-B19- β -globin, AAV-B19- α -globin, AAV-B19- γ -globin.
14. The vector of Claim 5 wherein said vector is
AAV-B19-mdr.
- 20 15. The vector of any of Claims 1-14 wherein
said vector is contained in a host cell.
16. The vector of any one of Claims 1-12 wherein
said vector is contained in a virion.
17. The vector of Claim 16 wherein said vector
25 is contained in a host cell.
18. The vector of Claim 15 or 17 wherein said
host cell is a hematopoietic stem or progenitor cell.
19. A method of use of the vector of Claim 1 for
gene therapy which comprises:
- 30 a) obtaining bone marrow cells from a patient;
 b) transducing said bone marrow cells with the
vector of Claim 1; and

1 c) reintroducing said transduced bone marrow
cells into said patient.

20. The method of Claim 19 wherein said
transducing is achieved by transfection with said vector
5 or by infection with virions containing said vector.

22. The method of Claim 19 wherein said gene
therapy comprises treatment of sickle-cell anemia or
diabetes.

23. The method of Claim 19 wherein said gene
10 therapy comprises treatment of thalassemia.

24. The method of Claim 23 wherein said vector
is AAV-B19- α -globin or AAV-B19- β -globin.

25. The method of Claim 19 wherein said gene
therapy comprises treatment of hematopoietic diseases.

15 26. The method of Claim 25 wherein said vector
is AAV-B19-GM-CSF.

27. The method of Claim 25 wherein said gene
therapy comprises treatment of cancer.

20 28. The method of Claim 27 wherein said vector
is AAV-B19-RB.

29. The method of Claim 19 wherein said gene
therapy comprises treatment of hemoglobinopathies.

30. The method of Claim 29 wherein said vector
is AAV-B19- β -globin, AAV-B19- α -globin or AAV-B19- γ -
25 globin.

31. A method of use of the vector of Claim 1 for
delivering a pharmaceutical product in a mammal which
comprises:

- 30 a) obtaining bone marrow cells from said mammal;
 b) transducing said bone marrow cells with the
vector of Claim 1 and

1 c) reintroducing said transduced bone marrow
cells into said mammal.

32. The method of Claim 31 wherein said
transducing is achieved by transfection or by infection
5 with virions containing said vector.

33. The method of Claim 31 wherein said product
is γ -globin, insulin, macrophage colony stimulating
factor, granulocyte colony stimulating factor,
erythropoietin, tumor necrosis factor, mast cell growth
10 factor, any of interleukins 1-11, p53, adenosine
deaminase or an antisense RNA molecule.

34. The method of Claim 31 wherein said product
is granulocyte macrophage-colony stimulating factor.

35. The method of Claim 34 wherein said vector
15 is AAV-B19-GM-CSF.

36. The method of Claim 31 wherein said product
is α -globin.

37. The method of Claim 36 wherein said vector
is AAV-B19- α -globin.

38. The method of Claim 31 wherein said product
20 is β -globin.

39. The method of Claim 38 wherein said vector
is AAV-B19- β -globin.

40. The method of Claim 31 wherein said product
25 is retinoblastoma.

41. The method of Claim 40 wherein said vector
is AAV-B19-RB.

42. A method of use of the vector of Claim 11
for conferring cell-specific multidrug resistance to a
30 patient which comprises:

a) obtaining bone marrow cells from a patient;

1 b) transducing said bone marrow cells with the
vector of Claim 11; and

 c) reintroducing said transduced bone marrow
cells into said patient.

5 43. A method of use of the vector of Claim 14
for conferring multidrug resistance to erythroid cells
of a patient which comprises:

 a) obtaining bone marrow cells from a patient;

10 b) transducing said bone marrow cells with the
vector of Claim 14; and

 c) reintroducing said transduced bone marrow
cells into said patient.

15 44. The method of Claim 42 or 43 wherein said
transducing is achieved by transfection with said vector
or by infection with virions containing said vector.

 45. The method of any one of Claims 19, 31, 42
or 43 wherein said bone marrow cells are enriched for
stem cells before transduction.

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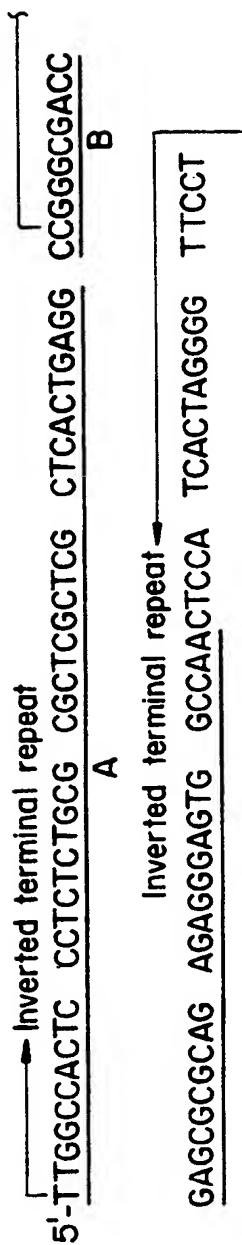


FIG. 1

2 / 20

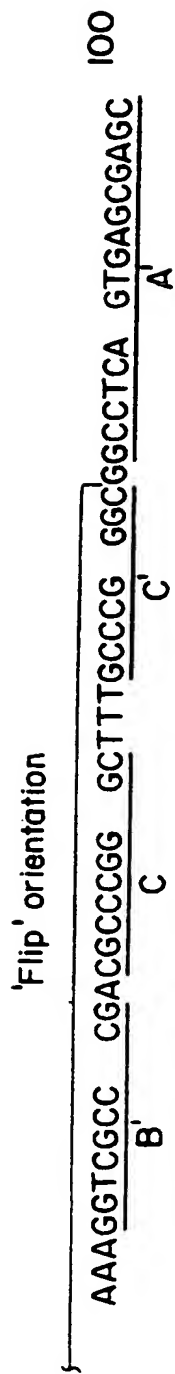


FIG. 1 CONT.

SUBSTITUTE SHEET

3 / 2 0

260
 ATTTTAAGTGTTTACTATAAATTTTATTGGTTAGTTTGTAAACGGTTAAAAATGGCGGAGCGTAGCGGGGA
 TAAAATTACACAAAATGATATTAAATAAACCAATCAAAACATTTGCCAATTTTACCCGCCCTCGCATCCGCCCT
 300
 MaeIII
 280
 380
 GCTTTTTCCTGGACTTTCCTTGCTGTTTTTTGTGAGCTAACTAACAGGTAATTTATACTACTTGT
 CGAAAGAGGACCTGAAAGAACGACACAAAACACATCGATTGATTGTCCATAAATATGATGAACAA
 420
 BstNI
 ScrFI
 AluI
 Hpa
 Hln

FIG. 2

4 / 2 0

220 TTTTAGCGGGCTTTTTCCTCCGCTTATGCAAAATGGGCAGCC 240
AAATCGCCCGAAAGAGGCGGAATACGTTTACCCGTCGG
BbvI

320 CTACAGTATATAGCACGGTACTGCCGCGAGCTCTTCTTTCTGGGCT 360
GATGTCATATATCGTGCCATGACGCGTCGAGAAAGAACCCCGA
RsaI BbvI

FIG. 2 CONT.

5 / 20

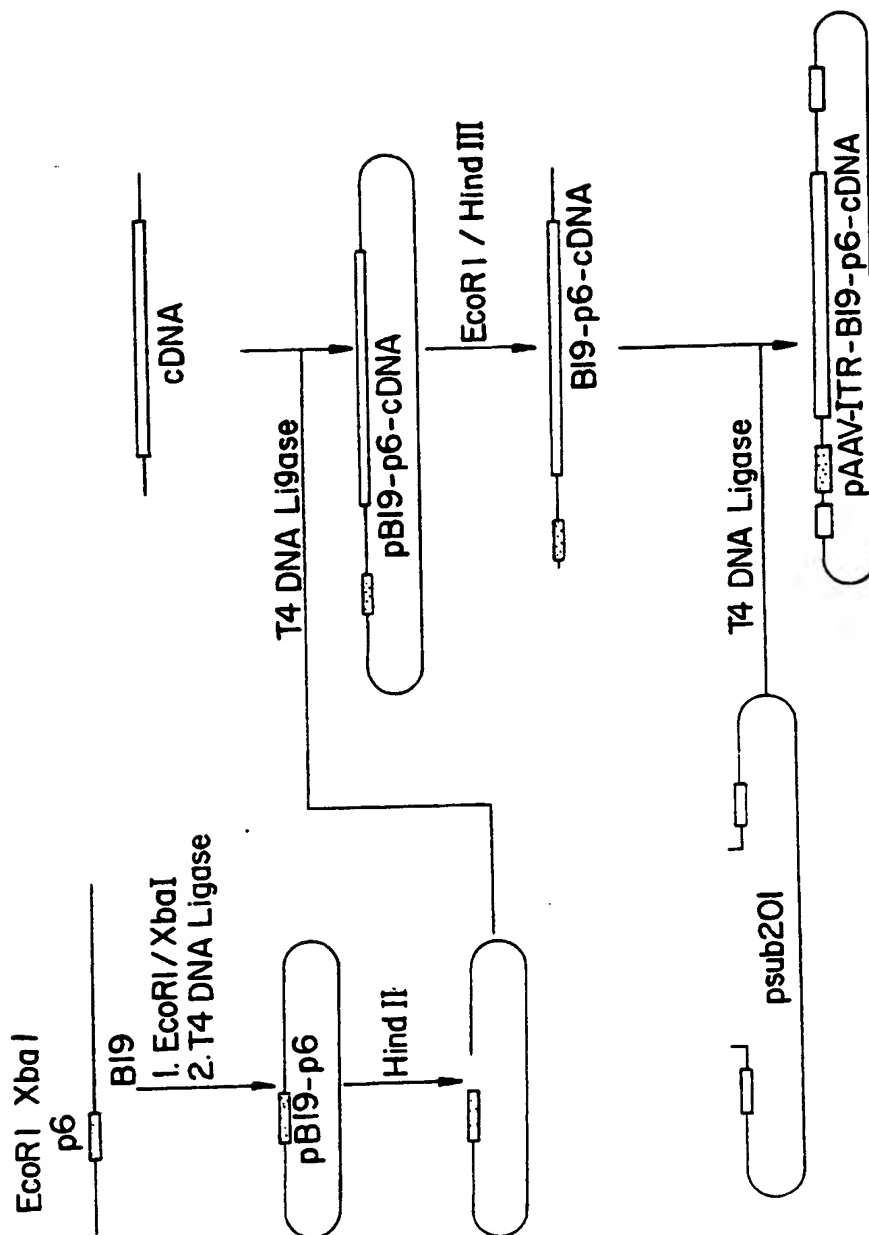


FIG. 3

6 / 20

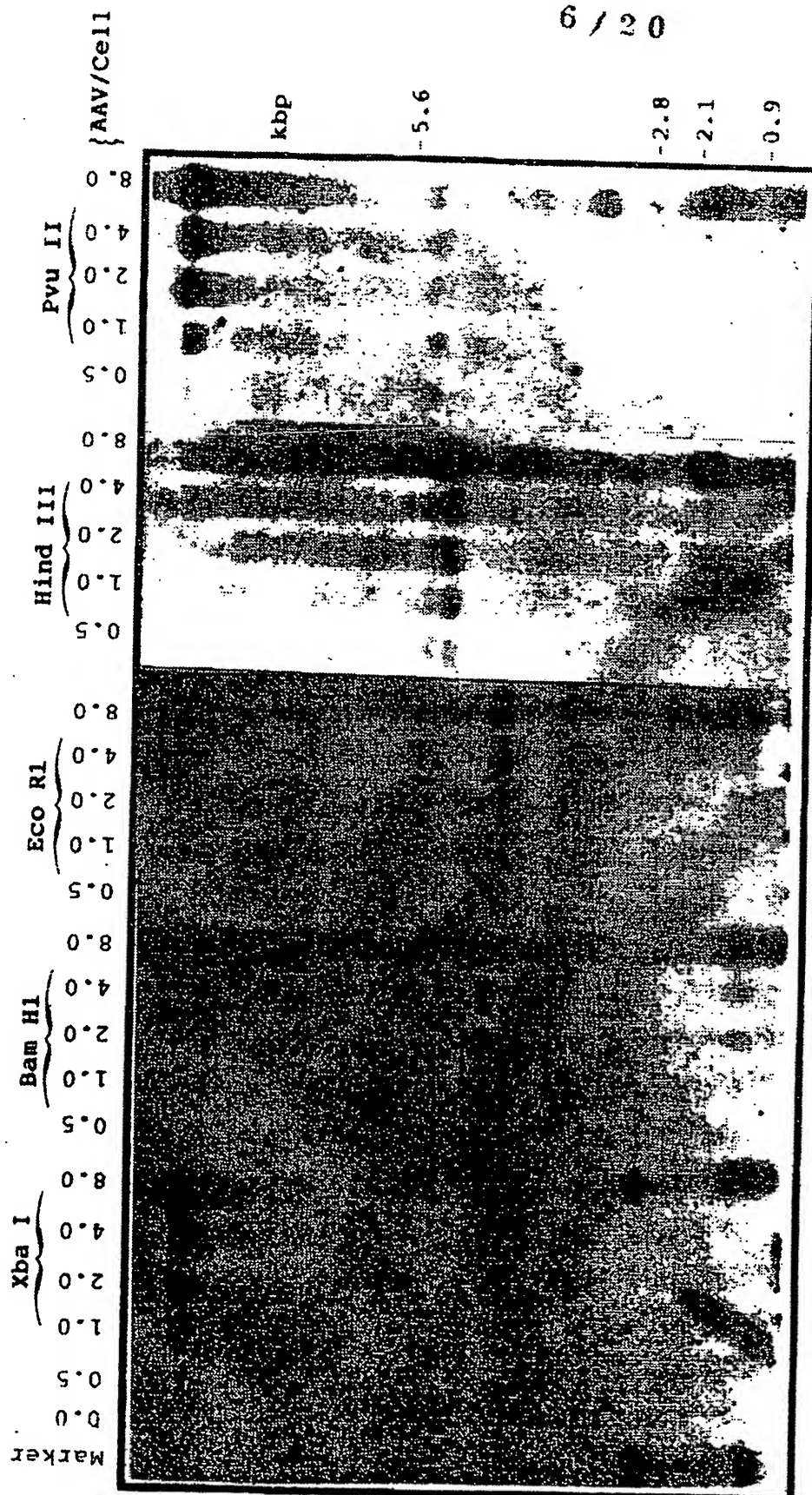
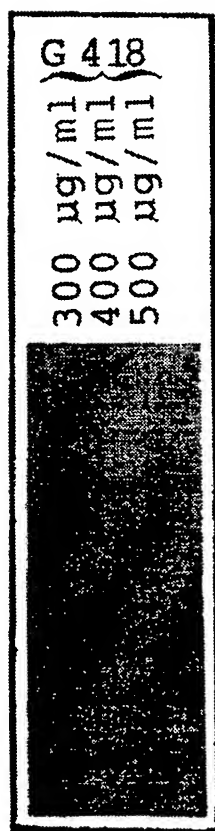


FIG. 4

7 / 20

FIG. 5



8 / 20

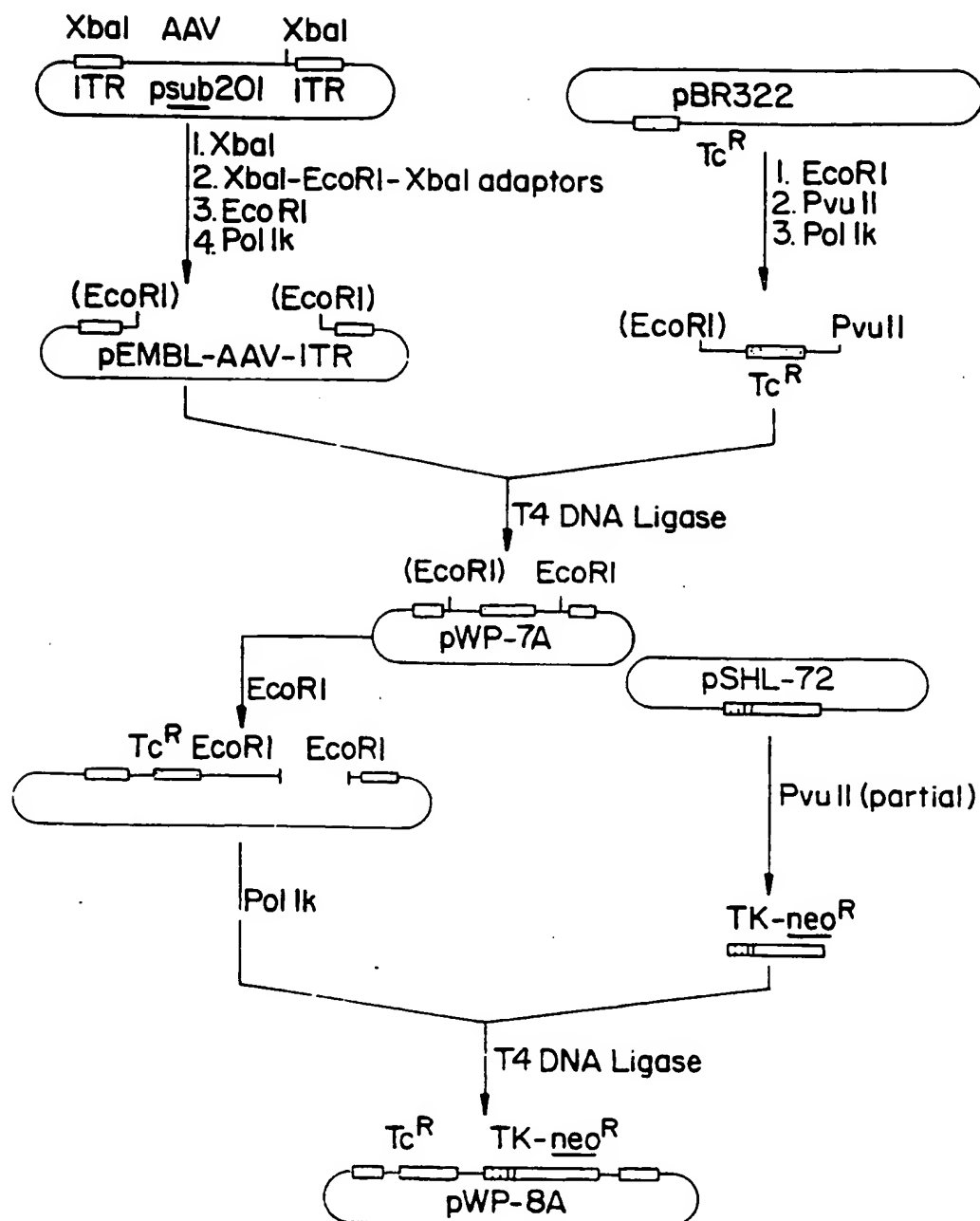


FIG. 6A

9 / 20

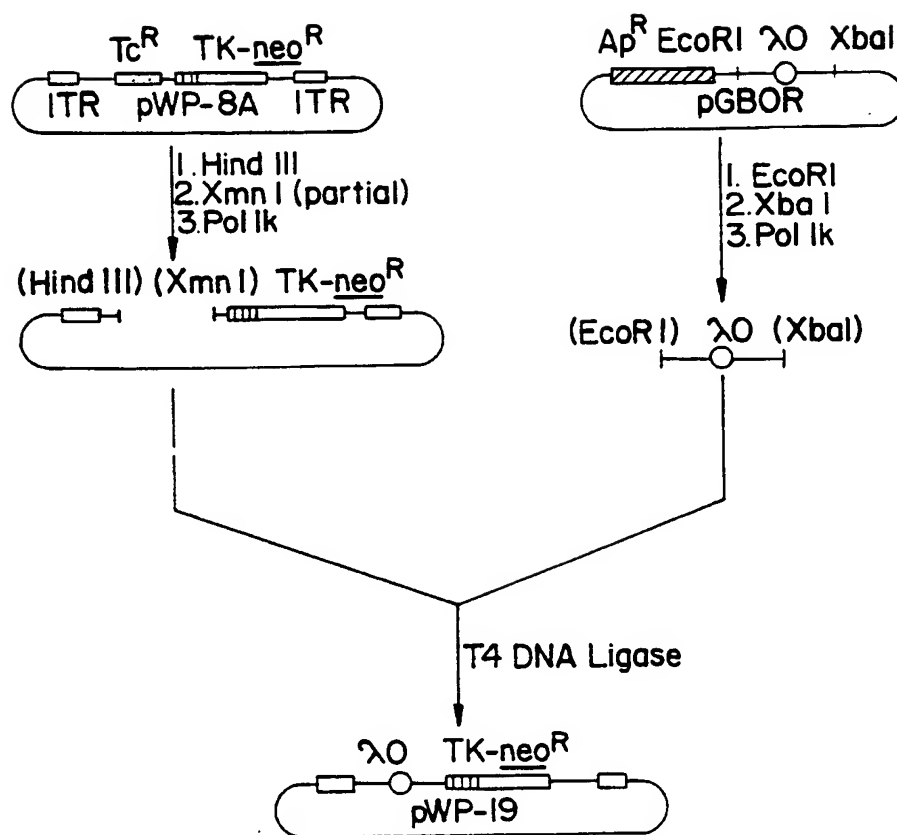


FIG. 6B

10 / 20

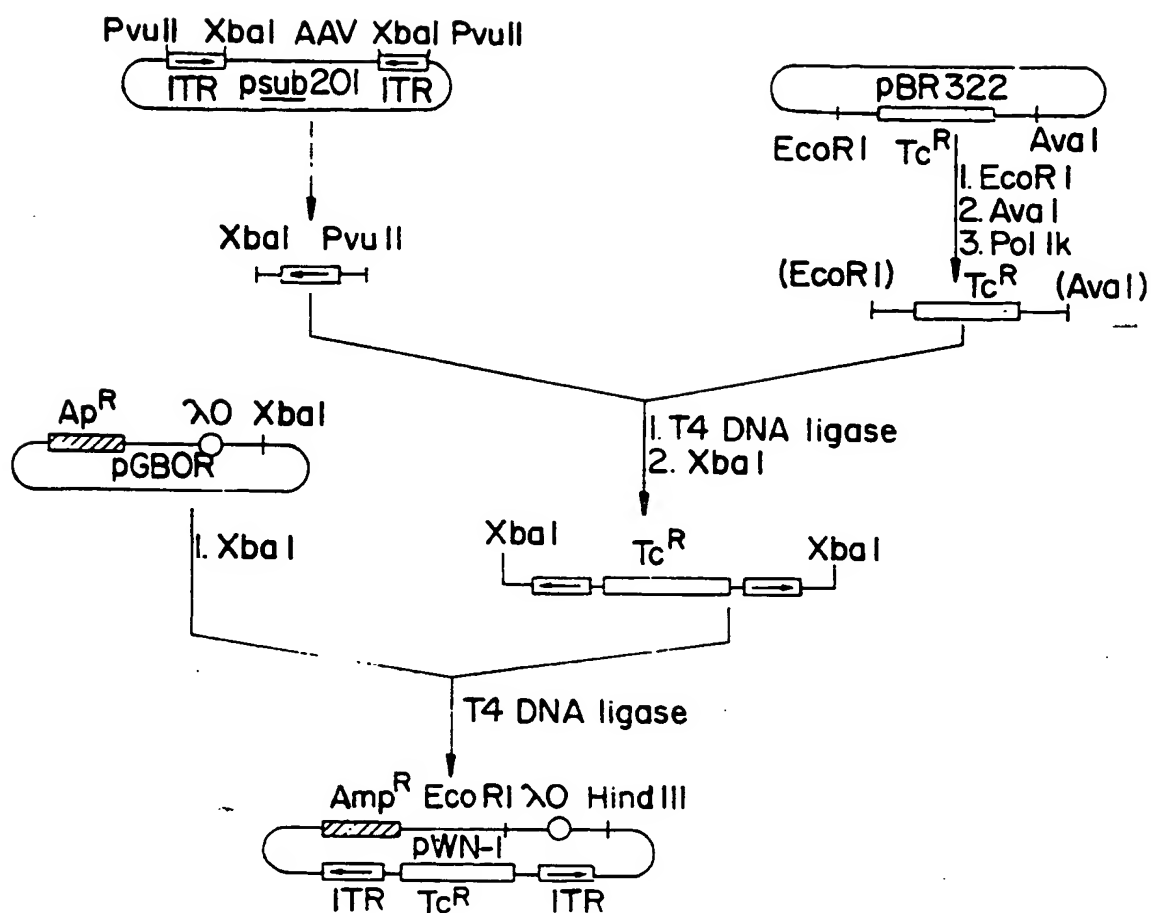


FIG. 7

11/20

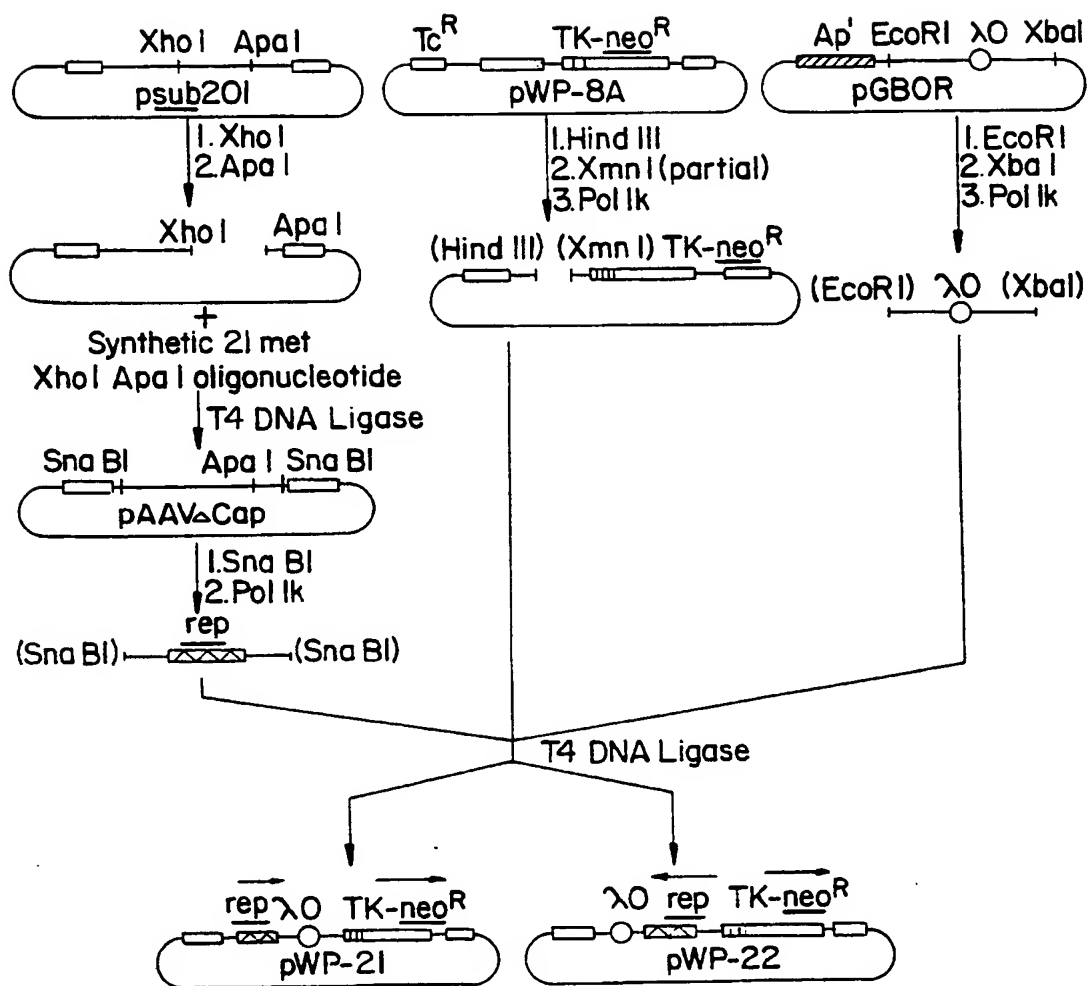


FIG. 8A

12/20

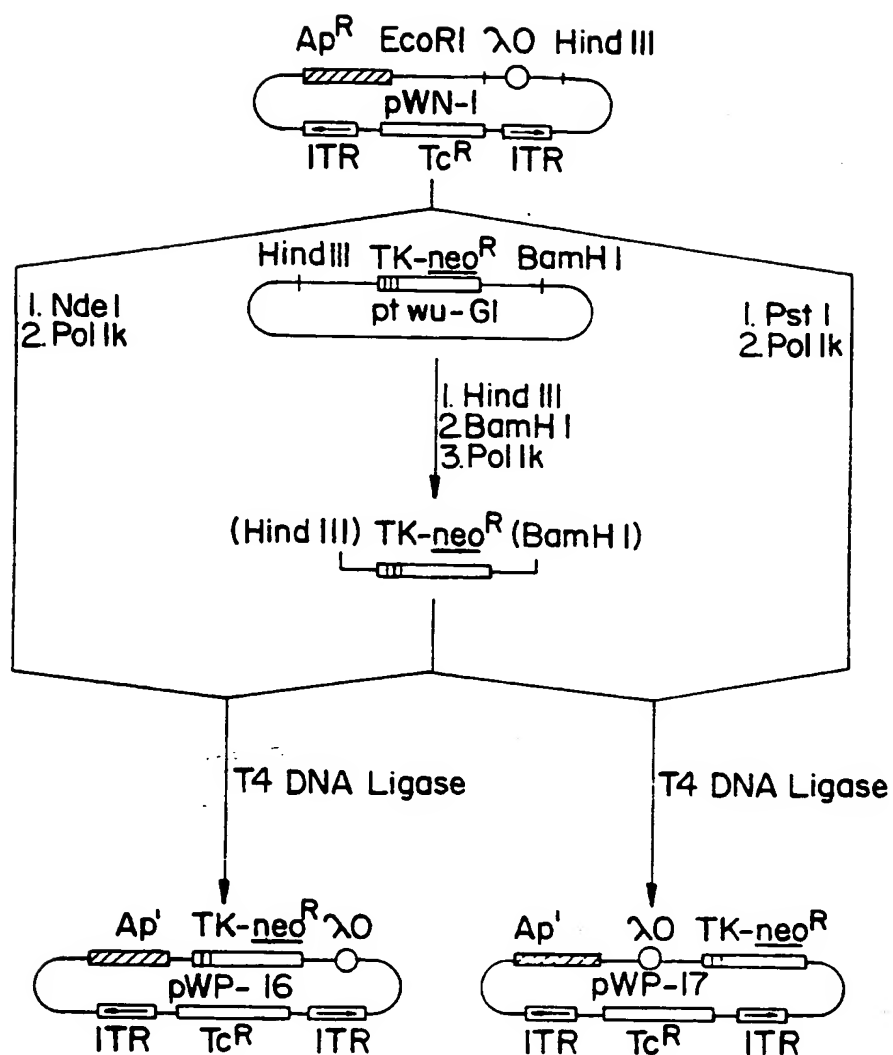
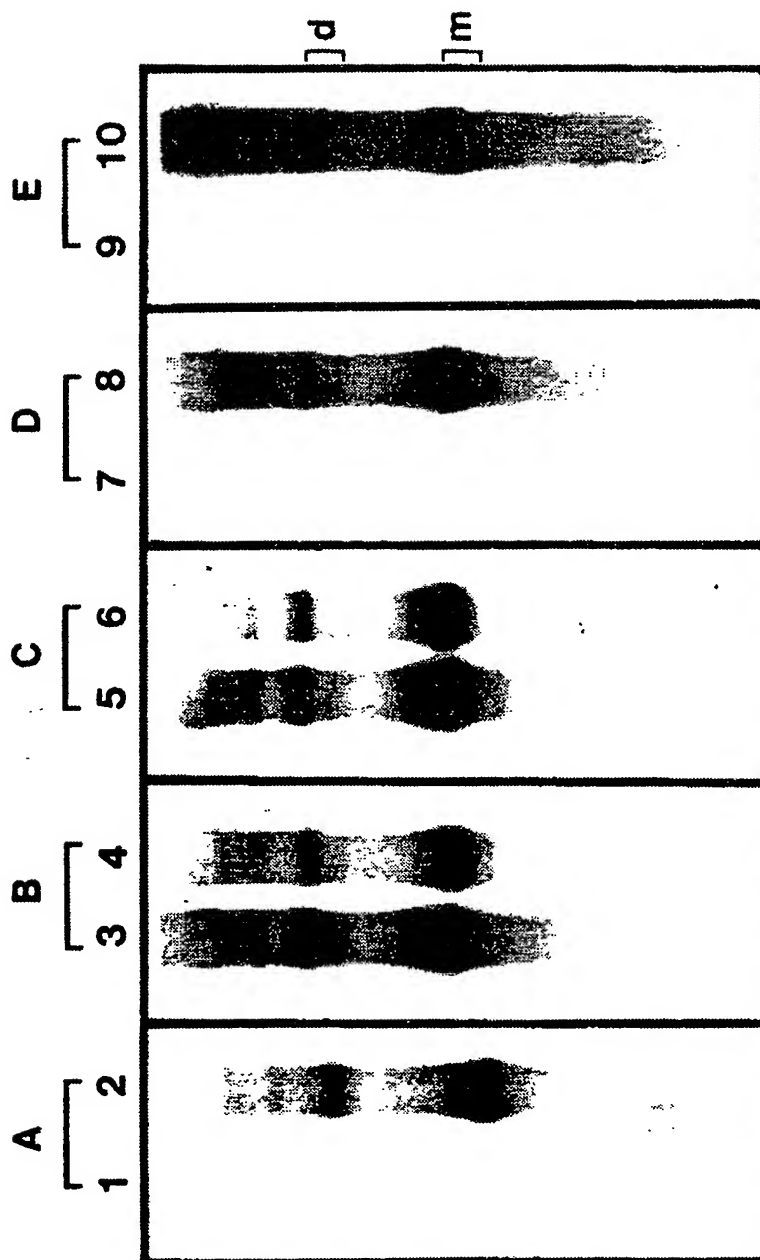


FIG. 8B

13 / 20

FIG. 9



SUBSTITUTE SHEET

14 / 20

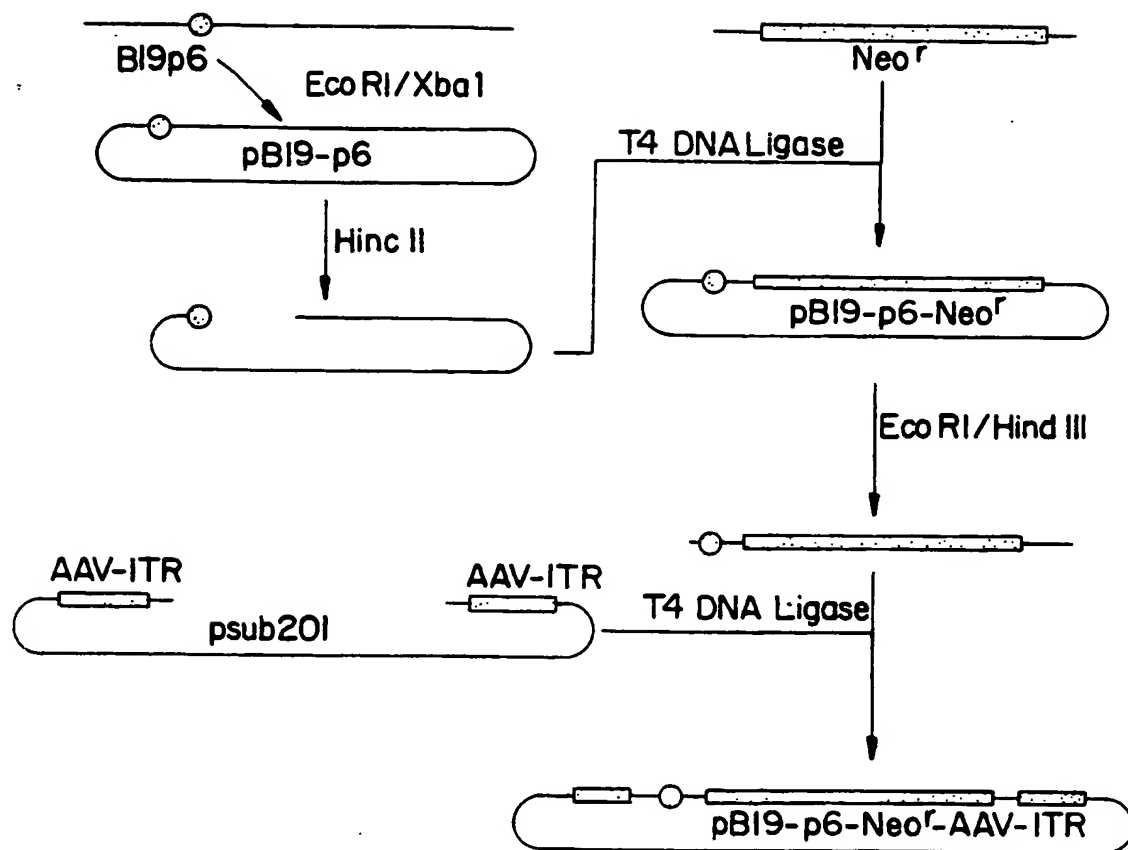


FIG. 10

15 / 20

AAV-mediated transfer of Neo^r to human hematopoietic stem cells.

A. Neo gene expression under the control of the TK promoter

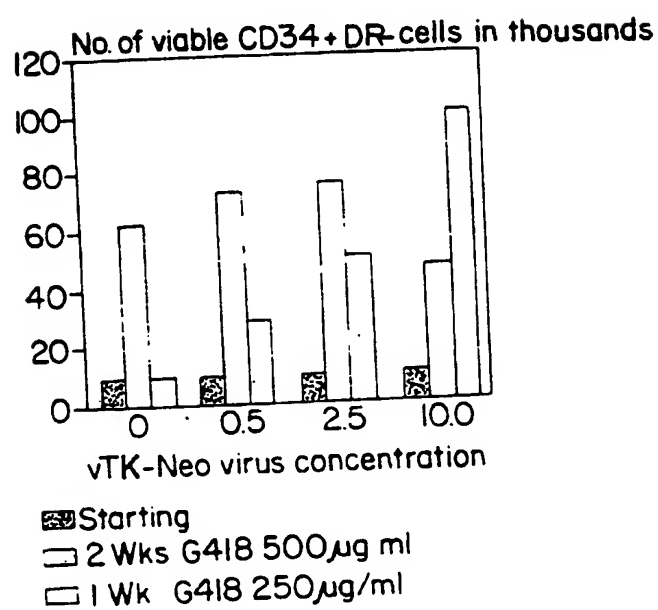


FIG. IIA

16 / 20

AAV-mediated transfer of Neo to
human hematopoietic stem cells.

B. Neo gene expression under the
control of the B19p6 promotor

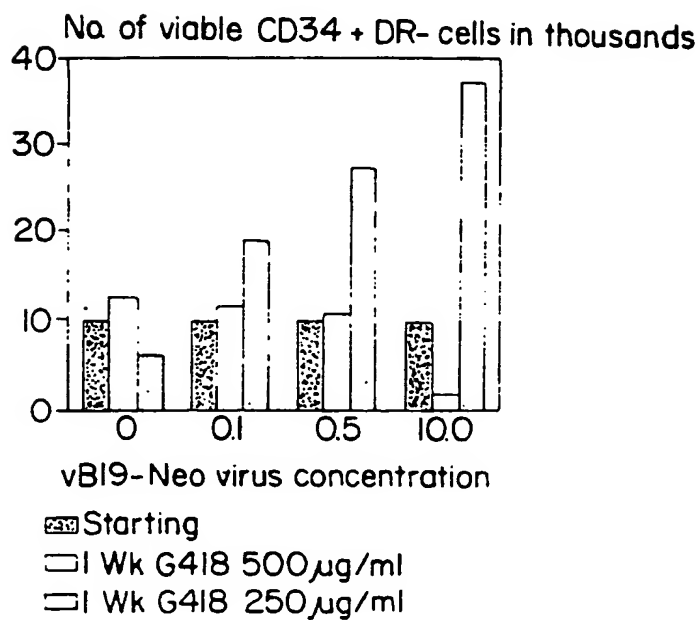


FIG. IIB

17 / 20

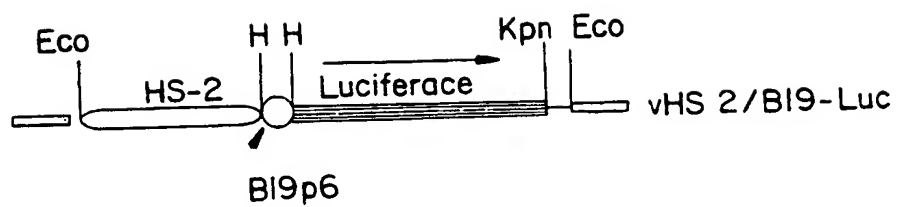


FIG. 12

18 / 20

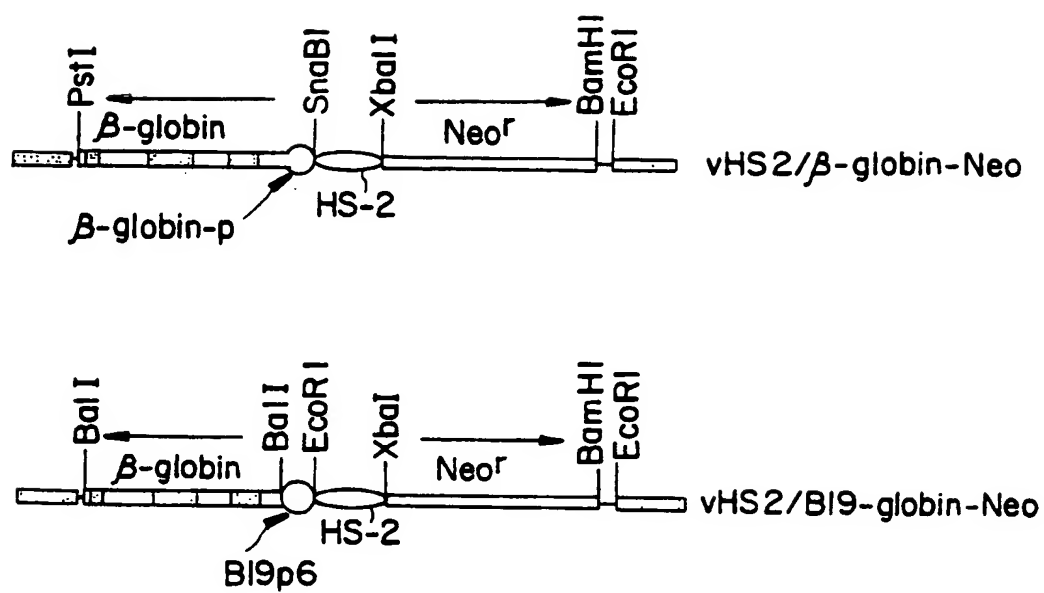


FIG. 13

19 / 20

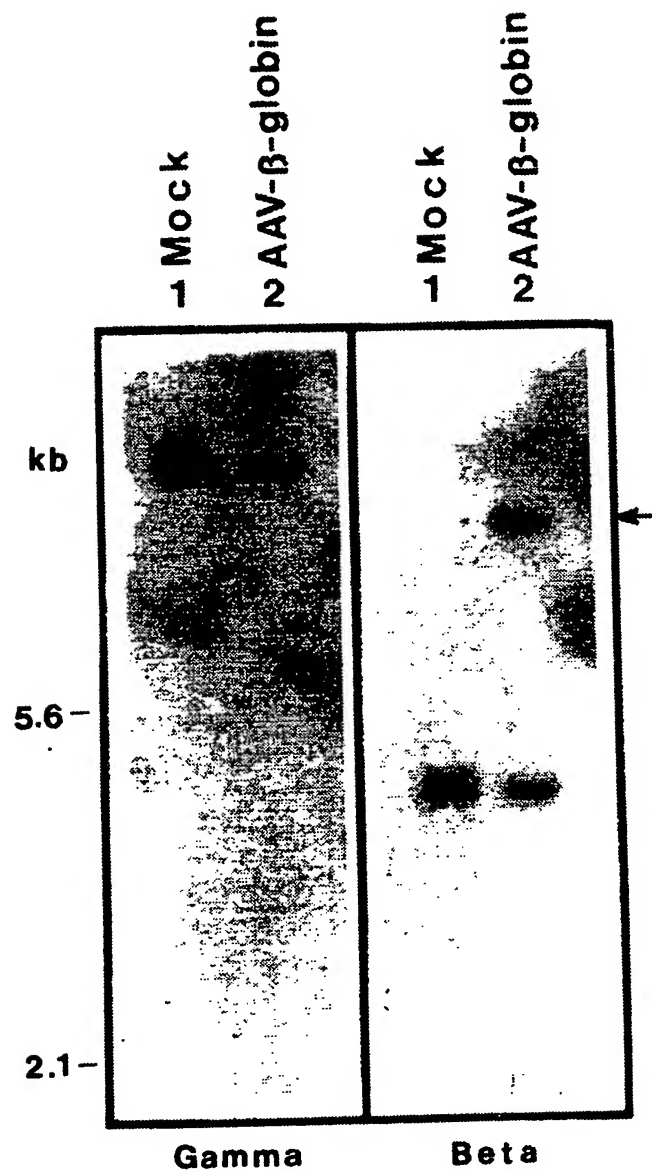


FIG. 14

SUBSTITUTE SHEET

20 / 20

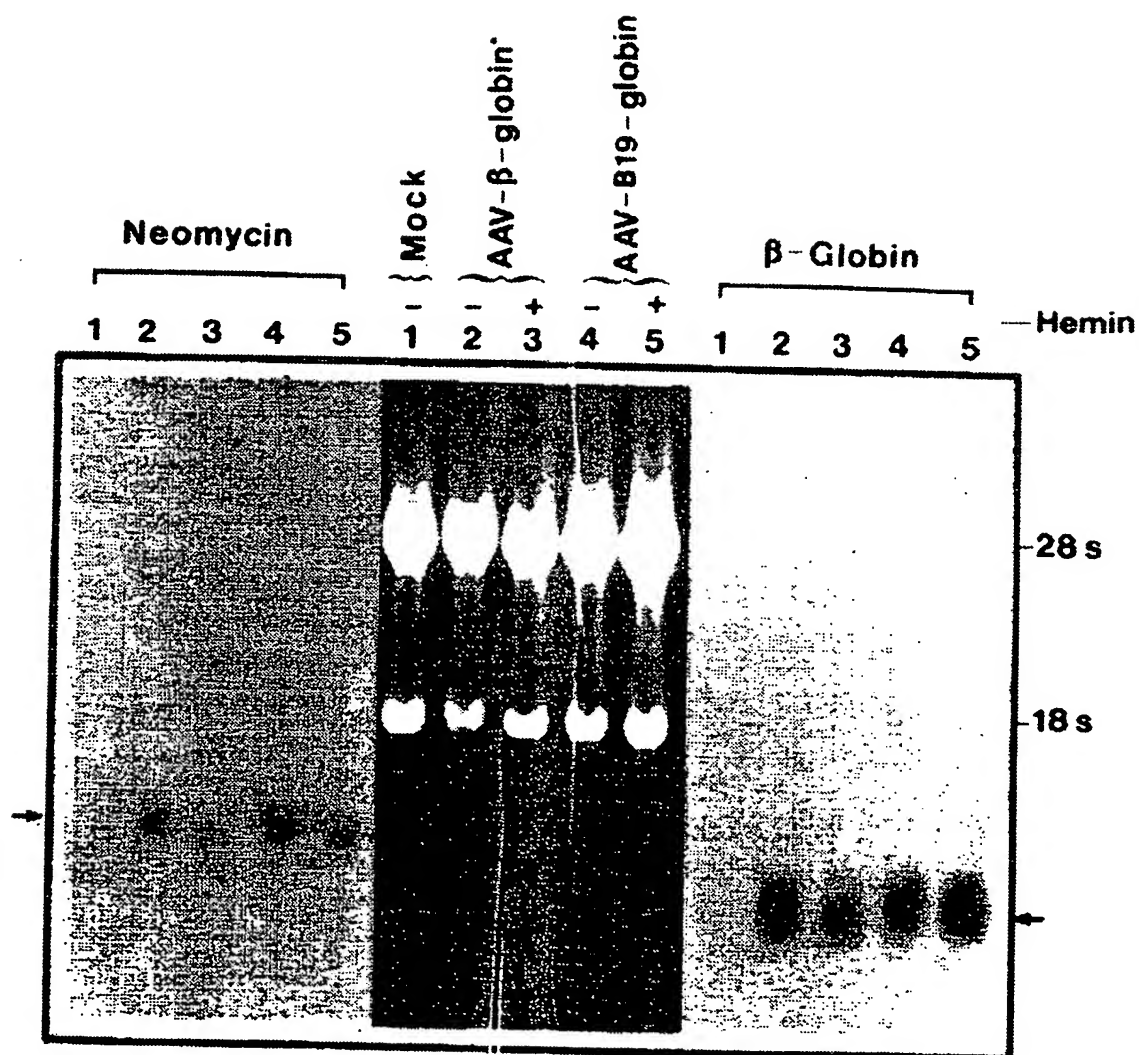


FIG. 15

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

PCT/US 92/09769

International Application No

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC Int.Cl. 5 C12N15/86; C12N15/12; A61K48/00								
II. FIELDS SEARCHED <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Minimum Documentation Searched⁷</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 30%; border-bottom: 1px solid black; padding: 5px;">Classification System</td> <td style="border-bottom: 1px solid black; padding: 5px;">Classification Symbols</td> </tr> <tr> <td style="padding: 5px;">Int.Cl. 5</td> <td style="padding: 5px;">C12N ; A61K</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched⁸</div>			Classification System	Classification Symbols	Int.Cl. 5	C12N ; A61K		
Classification System	Classification Symbols							
Int.Cl. 5	C12N ; A61K							
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹ <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%; border-bottom: 1px solid black; padding: 5px;">Category¹⁰</th> <th style="width: 60%; border-bottom: 1px solid black; padding: 5px;">Citation of Document,¹¹ with indication, where appropriate, of the relevant passages¹²</th> <th style="width: 30%; border-bottom: 1px solid black; padding: 5px;">Relevant to Claim No.¹³</th> </tr> <tr> <td style="vertical-align: top; padding: 5px; text-align: center;">X</td> <td style="padding: 5px;"> WO,A,8 808 450 (FINLAYSON , PECK) 3 November 1988 see page 5, line 5 - page 7, line 32; claims 1-89; figure 1 see page 34, line 15 - page 36, line 25 see page 52, line 22 - page 59, line 10 <div style="text-align: center;">---</div> <div style="text-align: center;">-/--</div> </td> <td style="vertical-align: top; padding: 5px;"> 1-3,7, 10,15, 18-20, 31,32,42 </td> </tr> </table> <div style="padding: 10px;"> <div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁰ Special categories of cited documents : ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div> </div>			Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	X	WO,A,8 808 450 (FINLAYSON , PECK) 3 November 1988 see page 5, line 5 - page 7, line 32; claims 1-89; figure 1 see page 34, line 15 - page 36, line 25 see page 52, line 22 - page 59, line 10 <div style="text-align: center;">---</div> <div style="text-align: center;">-/--</div>	1-3,7, 10,15, 18-20, 31,32,42
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IV. CERTIFICATION <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;"> Date of the Actual Completion of the international Search <div style="text-align: center; font-weight: bold;">15 FEBRUARY 1993</div> </td> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;"> Date of Mailing of this International Search Report <div style="text-align: center; font-weight: bold;">05.03.93</div> </td> </tr> <tr> <td style="border-bottom: 1px solid black; padding: 5px;"> International Searching Authority <div style="text-align: center; font-weight: bold;">EUROPEAN PATENT OFFICE</div> </td> <td style="border-bottom: 1px solid black; padding: 5px;"> Signature of Authorized Officer <div style="text-align: center; font-weight: bold;">HORNIG H.</div> </td> </tr> </table>			Date of the Actual Completion of the international Search <div style="text-align: center; font-weight: bold;">15 FEBRUARY 1993</div>	Date of Mailing of this International Search Report <div style="text-align: center; font-weight: bold;">05.03.93</div>	International Searching Authority <div style="text-align: center; font-weight: bold;">EUROPEAN PATENT OFFICE</div>	Signature of Authorized Officer <div style="text-align: center; font-weight: bold;">HORNIG H.</div>		
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International Searching Authority <div style="text-align: center; font-weight: bold;">EUROPEAN PATENT OFFICE</div>	Signature of Authorized Officer <div style="text-align: center; font-weight: bold;">HORNIG H.</div>							

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category ^a	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	<p>GENE vol. 89, no. 2, 14 May 1990, ELSEVIER, AMSTERDAM, NL; pages 279 - 282 S. OHI ET AL. 'Construction and replication of an adeno-associated virus expression vector that contains human beta-globin cDNA' see page 279, line 1 - line 8</p>	1-3, 7, 10, 15
Y	<p>see page 279, left column, line 1 - page 280, left column, line 2 ---</p>	19-45
X	<p>VACCINES vol. 90, 1990, CSH LABORATORY PRESS, NEW YORK, US; pages 353 - 359 K.A. VINCENT ET AL. 'Replication and packaging of HIV envelope genes in a novel adeno-associated virus vector system' see page 353, line 1 - line 25 ---</p>	1-3, 15-17
Y	<p>PROC. NATL. ACAD. SCI. vol. 86, no. 20, October 1989, NATL. ACAD SCI., WASHINGTON, DC, US; pages 8078 - 8082 C.H. SRIVASTAVA ET AL. 'Construction of a recombinant human parvovirus B19: Adeno-associated virus 2 (AAV) DNA inverted terminal repeats are functional in an AAV-B19 hybrid virus' cited in the application see page 8078, left column, line 20 - line 22 see page 8081, right column, line 39 - line 45 ---</p>	1-11, 19-45
Y	<p>BLOOD vol. 76, no. 10, 15 November 1990, SAUNDERS, NEW YORK, US; pages 1997 - 2004 A. SRIVASTAVA ET AL. 'Parvovirus B19-induced perturbation of human megacaryocytopoiesis in vitro' cited in the application see page 1997, left column, line 1 - right column, line 15 ---</p>	1-11, 19-45
P, X	<p>WO, A, 9 118 088 (THE UNITED STATES OF AMERICA) 28 November 1991 see page 14, line 1 - page 23, line 28 -----</p>	1-3, 7-10, 15

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 92/09769

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 19-45 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

US 9209769
SA 67258

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

15/02/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-8808450	03-11-88	None	
WO-A-9118088	28-11-91	AU-A- 7906691	10-12-91

EPO FORM P0177

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

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